THE NUTRITIONAL REQUIREMENTS OF A STRAIN OF TYPE III PNEUMOCOCCUS[1]

ELIZABETH BADGER[2]

Department of Biological Chemistry, College of Medicine, University of Cincinnati, Cincinnati

Received for publication January 4, 1944

The present investigation of the nutrition of pneumococci arose from an attempt to develop a microbiological assay method for choline. In preliminary studies, the growth of several strains of pneumococci was tested in a modification of the synthetic medium of Rane and Subbarow (1940). Of the pneumococci studied, only one type III strain grew perceptibly and the growth of this organism was far from optimal. This observation suggested the need for a more detailed study of the nutritional requirements of the pneumococcus. Such a study has been made, and has led to the development of a semi-synthetic medium which supports luxuriant growth of the strain of type III pneumococcus mentioned above without loss of either virulence or type specificity.

EXPERIMENTAL

Organism. The present study was concerned principally with the nutrition of the CHA strain of type III pneumococcus. This strain was obtained from Dr. L. H. Schmidt at the Christ Hospital Institute for Medical Research, Cincinnati. The stock culture was grown in veal phosphate infusion broth and passed through mice three times weekly to maintain virulence.

Inoculum. The inoculum was prepared as follows. One drop of the stock culture was subcultured in 5 ml of veal phosphate broth enriched with 0.1 per cent glucose and incubated for 6 hours. Five ml of this culture were centrifuged and the cells washed with 5 ml of sterile basal medium by centrifugation and finally suspended in 5 ml of the sterile basal medium. In the early developmental work a relatively large inoculum was used (0.1 ml of the above suspension, equivalent to 1,000,000 pneumococci per ml of culture). In the later stages of the study a much smaller inoculum was used (0.002 ml of the above suspension, equivalent to 20,000 pneumococci per ml).

Measurement of growth. Growth was measured turbidimetrically at the end of 12, 18 and 24 hours incubation. In the majority of experiments a turbidity comparator described by Krebs, *et al.* (1942) was used as the measuring instrument. In some of the experiments the Klett-Summerson photoelectric colorimeter equipped with a 420 mμ filter was employed. With both instruments, the greater the turbidity the larger the readings. All tests were carried out in duplicate in matched pyrex test tubes. The turbidity readings reported in the various tables are averages of these duplicate tests. Due to unavoidable

[1] The data are taken from a dissertation submitted to the Faculty of the Graduate School, University of Cincinnati, June 1943, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Christ Hospital Fellow in Biological Chemistry, University of Cincinnati.

differences in inocula, the turbidimeter or colorimeter values at a given time varied in separate experiments. For this reason all readings in any one table represent values obtained with the same inoculum.

TABLE 1

A modification of Rane and Subbarow's medium for pneumococcus

Basal medium*	
Acid hydrolysate of Eastman de-ashed gelatin	6 gm
d(+) Glutamic acid	100 mg
l-Cystine	25 mg
KH_2PO_4	5 gm
Distilled water to make	800 ml
	Concentration per ml. medium
Biotin (Lederle concentrate)	0.002 μg
Calcium pantothenate	1.0 μg
Nicotinic acid	10.0 μg
Choline chloride	2.5 μg
Glucose†	5.0 mg
$MgSO_4 \cdot 7H_2O$†	1.0 mg
Riboflavin†	0.1 μg
Thioglycolic acid‡	16.0 μg

* pH adjusted to 7.8.

† Solution autoclaved separately for 10 minutes at 10 pounds pressure. Added to sterile medium aseptically.

‡ See footnote 3.

TABLE 2

Improved semi-synthetic medium for type III pneumococcus

Basal medium	
Vitamin-free casein hydrolysate	0.5 gm nitrogen
l-Cystine	0.025 gm
KH_2PO_4	5.0 gm
$MgSO_4 \cdot 7H_2O$	0.4 gm
Distilled water to make	800 ml
	Concentration per ml. medium
Choline chloride	5.0 μg
Calcium pantothenate	1.0 μg
Nicotinic acid	1.0 μg
Biotin (crystalline)	0.002 μg
Thiamine	2.0 μg
Creatine	5.0 μg
Asparagine	1.0 μg
Ascorbic acid	300 μg
Glucose*	5.0 mg

* Autoclaved separately and added to sterile medium aseptically.

Media. The synthetic medium of Rane and Subbarow (table 1), to which biotin concentrate and thioglycolic acid[3] were added, served as a starting point

[3] The thioglycolic acid solution was prepared daily by adding 0.2 ml of concentrated thioglycolic acid to 100 ml of sterile distilled water.

in this study. This medium supported slow growth of the CHA strain. Quantitative and qualitative examination of the requirements of this organism for each of the constituents of the Rane and Subbarow medium and for certain other substances resulted in the development of a much improved medium (table 2), which supported rapid growth of the CHA strain.

The essential details of the preparation of the improved medium are as follows. In preparing the basal medium, the cystine was dissolved in the acid hydrolysate by warming the mixture slightly. After adding the inorganic salts and water, the pH was adjusted to 7.8 with concentrated NaOH. This mixture was heated to boiling and immediately filtered to remove insoluble phosphates. The basal medium was prepared at least two days before it was to be used and was stored in the refrigerator. This aging of the basal medium shortened the lag period considerably. The growth factors were made up to one hundred times the desired concentration in water solution and added to 8.0 ml portions of the basal medium in the concentrations given in table 2. The ascorbic acid was weighed and added to the medium just before it was to be tubed. The total volume in each tube was made up to 9.8 ml with distilled water and the tubes autoclaved for 15 minutes at 10 pounds pressure. A 25 per cent glucose solution was autoclaved separately and 0.2 ml added aseptically to each tube. After a thorough mixing of the glucose, the tubes were immediately inoculated. It was found that the sterile medium could be stored in the refrigerator for two or three days without reducing its growth capacity.

THE IMPORTANCE OF COMPONENT PARTS OF THE MEDIUM

Source of amino acids. It was observed in early experiments that hydrolyzed casein supported better growth than gelatin. The best growth was obtained with 0.5 mg nitrogen per ml medium of a hydrolysate prepared from SMACO vitamin-free casein. This hydrolysate was prepared by refluxing the casein for 18 hours in 8N HCl, removing the excess HCl by vacuum distillation and subsequently decolorizing with Darco charcoal. A hydrolysate prepared by Mueller's method (1941), in which the excess HCl was removed with PbO, as well as SMACO vitamin-free casein hydrolysate gave comparable results. Casamino acids (Difco) also provided a good source of amino acids but contained considerable quantities of choline.

The casein hydrolysate did not contain sufficient cystine for optimal growth. The addition of 20 to 25 micrograms per ml medium, the same quantity used by Rane and Subbarow, gave best results. As shown in table 3, cystine could be replaced by cysteine and glutathione but not by methionine, homocysteine or thioglycolate. This observation is of interest in connection with the function of cystine, and suggests that this compound functions as an essential amino acid rather than as a non-specific source of organic sulfur or a source of the sulfhydryl group.

Rane and Subbarow found glutamic acid an essential component of their medium. It was not necessary to add glutamic acid to our medium, probably because casein contains 21.7 per cent in contrast to 5.8 per cent in gelatin (Mathews, 1939).

Salt requirement. Both KH_2PO_4 and $MgSO_4$ were required for growth but their concentrations were not critical. The optimum concentrations were around 5 mg KH_2PO_4 per ml medium and around 0.3 to 0.4 mg $MgSO_4$ per ml. The concentration of KH_2PO_4 was the same as that used by Rane and Subbarow but the $MgSO_4$ concentration was less than half. With this lower concentration it was possible to include the $MgSO_4$ in the basal medium and still retain a clear medium after autoclaving. Using 0.4 mg of $MgSO_4$ and 5.0 mg KH_2PO_4 per ml of medium, the total concentration of the inorganic salts including the NaCl

TABLE 3

The effect of sulfur compounds on the growth of a type III pneumococcus

SULFUR COMPOUND	MICROGRAMS PER ML	TURBIDIMETER READINGS		
		12 hrs	18 hrs	24 hrs
Cystine	0	36	48	68
	20	57	72	113
Cysteine	10	49	56	100
	20	57	70	110
	30	57	71	114
	40	55	69	113
Glutathione	30	56	67	113
	60	54	63	108
	90	54	60	105
	120	53	60	103
Homocysteine	15	37	48	78
	25	38	47	77
	35	37	47	78
	50	38	47	78
Methionine	15	36	43	64
	25	36	43	63
	50	37	44	65
Sodium thioglycolate	5	39	47	75
	10	37	49	75
	20	39	55	87

of the hydrolysate was about 0.05 molar, well within the limit of 0.1 molar determined by Dernby and Avery (1918).

Reducing agent. Dubos (1929) has shown that the oxidation-reduction potential of the medium is highly important for growth of pneumococci. Thioglycolic acid, glutathione and ascorbic acid were tried as possible reducing agents in producing the desired potential. The best results were obtained with ascorbic acid when added to the medium in concentrations equal to 300 micrograms per ml medium. The addition of the ascorbic acid to the medium before autoclaving not only decreased the lag period but produced more consistent growth in duplicate tubes.

Essential growth-factor requirements. Pantothenic acid, nicotinic acid and choline were found by Rane and Subbarow to be essential for the growth of certain strains of pneumococcus. A. A. Andersen[4] had found that biotin was also required. Our observations and also those of Landy, *et al.* (1942) confirm this requirement. It was later learned that Rane[4] had also found biotin to be required for the growth of all strains tested. The response of the CHA strain to the four growth factors is shown in figure 1. Each curve represents a separate

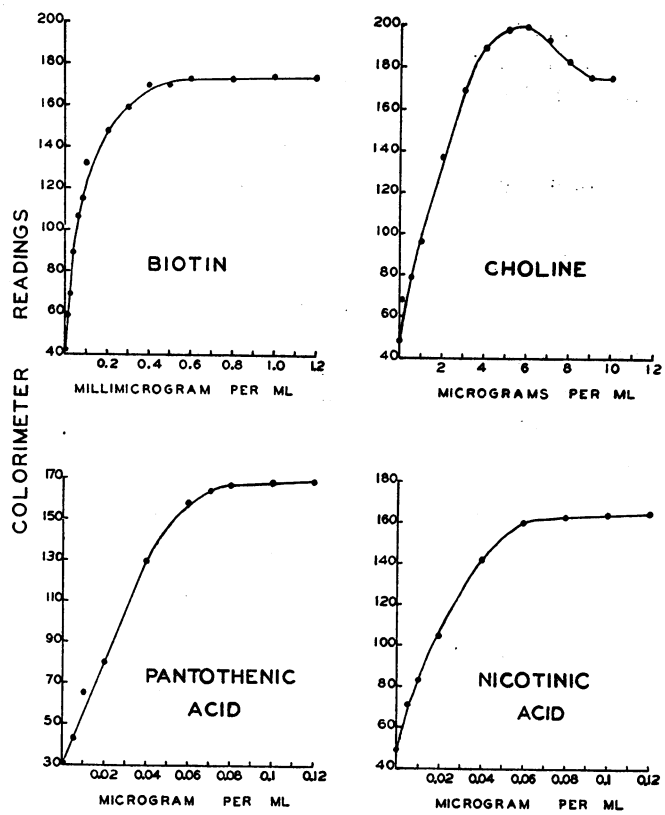

FIG. 1. RESPONSE OF TYPE III PNEUMOCOCCUS TO BIOTIN, CHOLINE, PANTOTHENIC ACID AND NICOTINIC ACID

Concentrations of choline and pantothenic acid given as choline chloride and calcium pantothenate respectively.

experiment so that the maximum turbidities are not comparable. The medium as given in table 2 was used and growth was measured after 15 hours incubation at 37.5 C.

The addition of as little as 0.00001 microgram of biotin to the medium stimulated growth; 0.0006 microgram produced maximum growth and concentrations as high as 0.01 microgram per ml had no inhibiting effect. Crystalline biotin could be replaced with the biotin methyl ester but not by pimelic acid.

[4] Personal communication.

The effect of varying concentrations of nicotinic acid on the growth of this type III pneumococcus is seen in figure 1. A minimum concentration of 0.08 microgram of nicotinic acid per ml of medium supported maximum growth. Concentrations as high as 20 micrograms per ml produced no inhibition. The nicotinic acid could be replaced with nicotinamide.

The results of a typical pantothenic acid titration are shown in figure 1. Maximum growth was obtained with 0.08 microgram per ml and no inhibition was observed with concentrations as high as 5 micrograms per ml. β-alanine could not replace pantothenic acid, which is in agreement with the observations of Rane and Subbarow (1940) that hydrolysis of the pantothenic acid molecule prevented growth of the pneumococcus.

TABLE 4

Growth response of a type III pneumococcus to ethanolamine or choline

ADDITION	COLORIMETER READINGS	
	15 hrs	24 hrs
Choline chloride		
micrograms per ml		
0.0	48	54
0.1	67	79
1.0	96	151
3.0	169	232
5.0	198	280
Ethanolamine		
3.0	50	73
6.0	85	160
12.0	119	173
15.0	136	181
18.0	152	198
24.0	161	208
30.0	166	220

Repeated choline titrations have shown the optimum concentration of choline to be between 5 to 6 micrograms per ml medium, as indicated in figure 1. Concentrations greater than this show inhibition. This rather limited optimum concentration was not changed after 24 subcultures in the semi-synthetic medium, nor was it affected by increasing the concentrations of the other growth factors. It is interesting to note the relatively high choline requirement in contrast to the other growth factors, especially since a similar relation holds in animal nutrition.

In a detailed study of the structural specificity of choline for the growth of this organism[5] it was found that ethanolamine was the only naturally occurring substance tested which could replace choline. A comparison of growth with choline and with ethanolamine is shown in table 4. From the results it is seen that maxi-

[5] To be reported in a separate paper.

mum turbidity obtained with ethanolamine was not equal to that obtained with choline, and that on a molecular basis approximately ten times as much ethanolamine was required to produce this maximum turbidity. However, as little as 6 micrograms of ethanolamine per ml medium stimulated growth of cultures with suboptimal concentrations of choline. These observations necessarily limit the use of the pneumococcus as a test agent in a microbiological assay of choline.

In cultures containing ethanolamine, the pneumococcus consistently grew in long chains, whereas in cultures containing ethanolamine plus choline or choline alone, no chains were formed. This chain formation persisted after 20 subcultures in the medium containing ethanolamine in place of choline. This observation is of interest in view of the report of Okamoto (1940) of characteristic long-chain growth of pneumococci grown on blood agar plates after the addition to the culture media of choline, tetramethylammonium iodide, tetraethylammonium iodide, diethylaminoethanol hydrochloride or triethanolamine hydrochloride among other amines.

Growth-accelerating factors. In experiments too numerous to record here, the effects of other accessory growth substances on the growth of this type III pneumococcus were studied. The compounds tested included adenine sulfate, adenylic acid, asparagine, β-alanine, carnitine, creatine, folic acid, glucoseamine, glutamine, glutathione, guanosine, inositol, methionine, nicotinamide, *p*-aminobenzoic acid, pimelic acid, phthiocol, pyridoxine, sarcosine, thiamine, tryptophane, uracil and urea. Of the substances tested, creatine (5 micrograms per ml), glutamine (150 micrograms per ml), and thiamine (2 micrograms per ml) accelerated growth and shortened the lag period. Later experiments showed that in the absence of glutamine, asparagine in a concentration of 1 microgram per ml accelerated growth to a greater extent than did glutamine.

It has been frequently pointed out that in any study of accessory growth substances, the conditions in which they produce a favorable action may be dependent not only on the limiting concentration of the substance itself but also upon the presence and quantity of the other medium constituents. Thus, quantitative results obtained with this medium might vary considerably if the concentrations of the various constituents were changed appareciably. This fact must be kept in mind in working out the specific amino acid requirements.

CHARACTERISTICS OF THE MEDIUM

Comparison of growth in the semi-synthetic medium with that in infusion broth enriched with blood. The classical medium for growth of pneumococci is meat infusion broth enriched with whole blood and serum or glucose or both. Figure 2 records a comparison of the growth of the CHA strain in the semi-synthetic medium with that in infusion broth enriched with 2 per cent defibrinated rabbit blood, the usual techniques for growth studies being employed. It is apparent from the data presented that growth in the two media is comparable, except for the prolonged lag phase in the semi-synthetic medium. Preliminary studies have shown that this lag period is almost abolished by the addition of peptone or yeast extract. This problem is being further investigated.

Capacity of the medium to support growth from small inocula. That the medium was essentially complete and not dependent upon unknown factors introduced by the inoculum was shown in several experiments. Cultures inoculated with 1 to 10 organisms from serial dilutions made in sterile basal medium showed maximum growth in 45 hours. Elliptical, opaque colonies appeared within 18 hours on plates made by adding 1.2 per cent Bacto agar to the semi-synthetic medium.

Capacity of the medium to support growth and maintain virulence after repeated transfers. To test further the completeness of the medium, a study of the growth and virulence of the organisms after repeated subcultures was undertaken. Two series of subcultures were made, one in the medium as given in table 2 and the

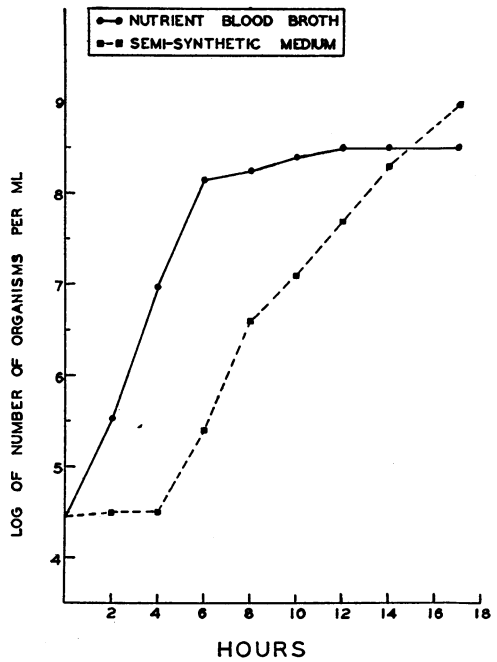

FIG. 2. GROWTH CURVES OF TYPE III PNEUMOCOCCUS GROWN IN NUTRIENT BLOOD BROTH AND SEMI-SYNTHETIC MEDIUM

other in the medium in which choline was replaced with 30 micrograms of ethanolamine per ml. To prevent excessive acid production, only 0.25 per cent glucose was used. The initial inoculum, containing about 20,000 organisms, was washed and diluted in sterile basal medium. The cultures were incubated 10 hours at 37.5 C and then placed in the refrigerator until the next subculture. Each succeeding subculture was made by inoculating 10 ml of the medium with 0.05 ml of the previous culture. After 20 subcultures, the rate, extent of growth and type specificity were the same as in the first subculture.

In table 5, the virulence of the organisms subcultured 20 times in the semi-synthetic medium containing choline or ethanolamine is compared with the virulence of the stock culture which has been passed through mice three times a

week. The control culture was a 12-hour subculture of mouse heart blood in nutrient broth enriched with 2 per cent rabbit blood. Twelve-hour cultures of the semi-synthetic media were also used. Serial dilutions of the cultures were made and the number of organisms was estimated by plate counts. Ten mice were used for each of the three highest dilutions and were injected intraperitoneally with one ml of the diluted cultures. The organisms subcultured in the medium containing choline appear slightly less virulent. This may be accounted for by the fact that the organisms grown in the nutrient broth and in the medium containing ethanolamine formed chains, thus producing a smaller colony count.

Capacity of the medium to support growth of other strains of pneumococci. In preliminary studies, five other strains of pneumococci, including two strains of type I, and one strain each of type II, III and VII, have been tested for growth in this medium. All five strains grew but showed a greater lag period than the type III (CHA), emphasizing again the differences in growth requirements of the various strains and types of pneumococci.

TABLE 5

Virulence of type III (CHA) pneumococcus after 20 subcultures in the semi-synthetic medium containing choline or ethanolamine

CONTROL CULTURE			AFTER 20 SUBCULTURES IN THE SEMI-SYNTHETIC MEDIA					
			Choline			Ethanolamine		
Culture dilution	Colony count	No. mice dead*	Culture dilution	Colony count	No. mice dead*	Culture dilution	Colony count	No. mice dead*
10^{-6}	308	10	10^{-6}	438	10	10^{-5}	680	10
10^{-7}	33	10	10^{-7}	41	9	10^{-6}	72	10
10^{-8}	3	8	10^{-8}	5	3	10^{-7}	7	9

* Number of mice dead out of 10 mice injected.

SUMMARY

The factors necessary for the growth of type III (CHA) pneumococcus were found to be a casein-acid-hydrolysate, cystine, KH_2PO_4, $MgSO_4$, nicotinic acid, pantothenic acid, biotin, choline, glucose, and ascorbic acid. The addition of creatine, asparagine or thiamine increased the rate of growth but was not essential. Cystine could be replaced with cysteine or glutathione but not with methionine, homocysteine or thioglycolic acid. Ethanolamine in ten times the molar concentration of choline could replace choline. Growth in the presence of ethanolamine occurred in characteristic long chains.

In the semi-synthetic medium described, the pneumococci were found not only to survive and multiply from very small inocula but to maintain their virulence and type specificity after repeated subcultures.

REFERENCES

DERNBY, K. G., AND AVERY, O. T. 1918 The optimum hydrogen ion concentration for the growth of pneumococcus. J. Exptl. Med., **28**, 345–357.

DUBOS, R. 1929 The initiation of growth of certain facultative anaerobes as related to oxidation-reduction processes in the medium. J. Exptl. Med., **49**, 559–573.

KREBS, R. P., PERKINS, P., TYTELL, A. A., AND KERSTEN, H. 1942 A turbidity comparator. Rev. Sci. Instruments, **13**, 229–232.

LANDY, M., DICKEN, D. M., BICKING, M. M., AND MITCHELL, W. R. 1942 Use of avidin in studies on biotin requirements of microorganisms. Proc. Soc. Exptl. Biol. Med., **49**, 441–444.

MATHEWS, A. P. 1939 *Physiological Chemistry*, p. 325. The Williams & Wilkins Co., Baltimore.

MUELLER, J. H., AND JOHNSON, E. R. 1941 Acid hydrolysates of casein to replace peptone in the preparation of bacteriological media. J. Immunol., **40**, 33–38.

OKAMOTO, HAJIME 1940 Characteristic long chain growth of pneumococci produced by quarternary ammonium compounds. Japan. J. Med. Sci. IV. Pharmacol., **12**, 143–156. Chem. Abstracts, **34**, 6319, 1940.

RANE, L., AND SUBBAROW, Y. 1940 Nutritional requirements of the pneumococcus. 1. Growth factors for types I, II, V, VII, and VIII. J. Bact., **40**, 695–704.