Nutritional Requirements of the Pneumococcus : I. Growth Factors for Types I, II, V, VII, VIII

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NUTRITIONAL REQUIREMENTS OF THE PNEUMOCOCCUS 1. GROWTH FACTORS FOR TYPES I, II, V, VII, VIII

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A study has been made of the nutritional requirements of the pneumococcus. The relatively simple, chemically-defined medium composed of glutathione, thiochrome, nicotinamide, betaine, flavin, glucosamine, uracil, guanylic acid, xanthine, hypoxanthine, pantothenic acid, gelatin hydrolysate, amino acids, inorganic salts, and glucose which can support the growth of the Dochez NY 5 strain of hemolytic streptococcus (Rane and Subbarow, 1938; Subbarow and Rane, 1939) was found to be deficient for pneumococci. The addition of a highly-active, purified extract of liver provided conditions suitable for good growth of certain types of *Pneumococcus*. One fraction isolated from this liver extract demonstrated the growth value of a compound similar to, and replaceable by choline. Satisfactory growth was subsequently obtained in a medium consisting of gelatin hydrolysate, certain additional amino acids, inorganic salts, glucose, choline, nicotinic acid, pantothenic acid, flavin, and thioglycollic acid. In some cases a mixture of known amino acids could be substituted for the gelatin hydrolysate.

EXPERIMENTAL

CULTURES

Pneumococci of Types I, II, V, VII and VIII have been used, one strain of each.[1] These have been passed through mice five

[1] These strains are used regularly at the Massachusetts Antitoxin and Vaccine Laboratory in mouse protection tests for the titration of potency of therapeutic antipneumococcic serum. We wish to thank our associates at the laboratory for their kind cooperation throughout this work.

days in each week, maintaining their virulence at a point where not more than five organisms are required to kill white mice within 72 hours when injected intraperitoneally.

To provide the inocula for these experiments, cultures obtained directly from the mouse were subcultured in a meat-infusion peptone broth containing 0.1 per cent glucose, incubated for six hours at 37°C., centrifuged, washed with sterile distilled water, and resuspended in sterile distilled water. The inoculations were made in 0.1 ml. amounts. Because of the rapid autolysis that pneumococci undergo, inoculated test substances were incubated at 37°C. for only 15 to 18 hours.

Growth was measured by direct readings of turbidity with a modified Gates Nephelometer; the heavier the growth, the lower the reading. Readings ranged from 2.3 to more than 4.7, the limit of direct reading. A reading of 4.7 or more indicates little, if any growth; a reading of 3.0 or less, good growth. Controls for the different types grown in a meat infusion peptone broth, gave these readings: Type I, 2.4; Type II, 2.3; Type V, 2.5; Type VII, 2.4; Type VIII, 2.4.

The experiments described below have been repeated several times. Variations in the nature of the inoculum resulted in slight differences in readings. The figures recorded in the following tables represent values obtained from single typical experiments.

MEDIA

Basal medium (1)

Acid-hydrolyzed Eastman de-ashed gelatin	6.0	grams
d-Glutamic acid	0.1	gram
l-Cystine	0.025	gram
KH_2PO_4	5.0	grams
Distilled water	800	ml.

Combined, pH adjusted with 5 N NaOH to 7.8, and tubed in 8.0 ml. amounts. Volume brought to 9.5 ml. with the addition of test substances and distilled water and autoclaved for 10 minutes at 112°C. 0.1 ml. 10 per cent $MgSO_4$ solution previously autoclaved separately for 10 minutes at 112°C., 0.1 ml. 50 per cent glucose solution also autoclaved separately, 0.1 ml. culture added to the test media. Final volume in all cases was made up to 10 ml.

The basal medium alone or combined with optimum concentrations of substances found favorable for the cultivation of the

Dochez NY 5 strain of hemolytic streptococcus did not permit growth of the strains used. However, the further addition to the latter medium of choline,[2] nicotinic acid, and thioglycollic acid produced good growth of some types even when glutathione, uracil, guanylic acid, xanthine, hypoxanthine, thiochrome, nicotinamide and glucosamine were omitted.

In preparing the final medium, the pantothenic acid,[3] nicotinic acid and choline were combined with the basal medium before autoclaving. To the sterile tubed medium were added flavin,

TABLE 1

Influence of combinations of various substances

SUBSTANCES ADDED TO BASAL MEDIUM NO. 1	NEPHELOMETER READINGS FOR TYPES:				
	I	II	V	VII	VIII
P.A.* + C. (or N.A., or T.A., or F.)	—‡	—	—	—	—
P.A. + C. + N.A. (or T.A., or F.)	—	—	—	—	—
P.A. + C. + N.A. + T.A.	2.7	2.5	3.4	—	2.5
P.A. + C. N.A. + F.	—	—	—	—	—
P.A. + C. + N.A. + T.A. + F.	2.4	2.2	3.0	—	2.2
P.A. + N.A. + T.A. + F.	—	—	—	—	—
Hydrolyzed P.A.† + C. + N.A. + T.A. + F.	—	—	—	—	—

* P.A. = Pantothenic acid 1 microgram/ml. medium; C. = Choline 2.5 micrograms/ml. medium; N.A. = Nicotinic acid 50 micrograms/ml. medium; T.A. = Thioglycollic acid 50 micrograms/ml. medium; F. = Flavin 0.1 microgram/ml. medium.

† Hydrolyzed by autoclaving with 0.1 N HCl for 30 minutes at 115°C.

‡ No growth.

separately autoclaved for 10 minutes at 112°C. and thioglycollic acid, previously sterilized by Berkefeld filtration. The volume was held constant at 10 ml.

Table 1 records the influence of the substances found essential for growth. With the exception of Type VII these factors in combination produced growth of all types tested; individually, the same substances were without effect. The substitution of

[2] Throughout these experiments reference is made to choline in the form of choline chloride.

[3] The pantothenic acid was assayed to be at least 50 per cent pure when compared with a preparation kindly supplied by Dr. R. J. Williams.

hydrolyzed for unhydrolyzed pantothenic acid destroyed the effectiveness of the combination.

The probability that gelatin hydrolysate includes substances still unknown made desirable its replacement by known amino acids. This medium was able to support the growth of Types II, V, and VIII but was deficient for the growth of Type I.

Basal medium (g)

d-Glutamic acid	1	gram
Glycine	0.25	gram
l-Asparagin	0.20	gram
d-l Leucine	0.15	gram
d-Arginine carbonate	0.075	gram
d-l Alanine	0.05	gram
d-l Lysine dihydrochloride	0.05	gram
d-l Methionine	0.05	gram
l-Cystine	0.05	gram
d-l Histidine monohydrochloride	0.025	gram
l-Tryptophane	0.025	gram
β-alanine	0.025	gram
Nor-leucine	0.015	gram
l-Phenylalanine	0.01	gram
l-Oxyproline	0.01	gram
KH_2PO_4	5	grams
NaCl	2.5	grams
Distilled Water	800	ml.

Combined, pH adjusted with 5 N NaOH to 7.8, and tubed in 8.0 ml. amounts. Volume brought to 9.5 ml. with the addition of test substances and distilled water and autoclaved for 10 minutes at 112°C. 0.1 ml. 10 per cent $MgSO_4$ solution previously autoclaved separately for 10 minutes at 112°C., 0.1 ml. 50 per cent glucose solution also autoclaved separately, 0.1 ml. culture added to the test media. Final volume in all cases was made up to 10 ml.

As in basal medium (1) the pantothenic acid, nicotinic acid and choline were combined before autoclaving while sterile thioglycollic acid and flavin were added after autoclaving.

To establish the optimum concentrations of the essential growth factors, experiments were done in which certain components were varied and others, held constant. Table 2 presents the effect upon growth of different concentrations of pantothenic acid, choline and nicotinic acid when thioglycollic acid and flavin are kept constant.

It appears then that Types I, II, V and VIII have somewhat different requirements for maximum growth. A marked inter-

dependence between the three variables is evident. The optimum concentration of choline, about the same for all types tested, was somewhat limited in range. In the presence of sufficient amounts of choline and pantothenic acid, the optimum for nicotinic acid

TABLE 2

*Influence of varying concentrations of pantothenic acid, choline and nicotinic acid**

NICOTINIC ACID	CHOLINE	NEPHELOMETER READINGS FOR TYPES:									
		I			II			V		VIII	
		Pantothenic acid micrograms/ml.									
		1	0.5	0.25	1	0.5	0.25	1	0.5	1	0.5†
micrograms/ml.	*micrograms/ml.*										
50	10	2.9	3.7	>4.7	2.4	4.0	>4.7	3.8	>4.7	3.8	>4.7
50	5	2.5	2.8	2.8	2.4	2.8	>4.7	3.5	>4.7	3.2	>4.7
50	2.5	2.4	2.4	2.5	2.4	2.4	2.5	3.4	4.6	2.8	>4.7
50	1	3.4	3.3	3.4	3.3	3.5	3.6	4.7	>4.7	3.0	>4.7
50	0.5	4.2	4.3	4.5	4.0	4.1	4.6	>4.7	>4.7	>4.7	4.7
10	10	3.3	4.2	>4.7	2.5	4.0	>4.7	>4.7	>4.7	4.4	>4.7
10	5	2.7	2.9	3.0	2.3	2.8	>4.7	3.5	>4.7	4.3	>4.7
10	2.5	2.7	2.7	3.7	2.4	2.4	2.5	3.4	4.6	3.4	>4.7
10	1	3.7	3.7	3.9	3.5	3.6	3.6	>4.7	>4.7	>4.7	>4.7
10	0.5	4.4	4.6	>4.7	4.1	4.3	4.7	>4.7	>4.7	>4.7	>4.7
2	10	3.7	4.5	>4.7	2.5	4.1	>4.7	>4.7	>4.7	>4.7	>4.7
2	5	2.7	3.0	3.3	2.4	2.9	>4.7	3.5	>4.7	>4.7	>4.7
2	2.5	3.0	3.0	3.9	2.4	2.6	2.6	3.6	>4.7	3.5	>4.7
2	1	3.8	3.9	4.2	3.6	3.8	3.9	>4.7	>4.7	>4.7	>4.7
2	0.5	4.7	>4.7	>4.7	4.5	4.6	4.7	>4.7	>4.7	>4.7	>4.7

* Type I grown in basal medium (1). Types II, V, VIII grown in basal medium (2). Constant in all tubes: Thioglycollic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.

† Pantothenic acid 0.25 microgram/ml. gave readings of >4.7 with Types V and VIII throughout.

was the same for all types. The optimum concentration of pantothenic acid for the types studied differed when the amounts of choline and nicotinic acid were varied.

The relationship between the concentrations of pantothenic acid and choline is well demonstrated in table 3 with Type II as

the test organism. Increasing the amount of choline necessitated a relative increase in the concentration of the pantothenic acid to obtain equivalent growth.

The need for a sulphydryl reducing substance early became apparent. This is not surprising in view of the importance of such an agent in the cultivation of various types of streptococci in chemically defined media. Several compounds were tried including glutathione, ascorbic acid, and thioglycollic acid; and the latter proved to be satisfactory. That growth does not take place in the absence of this reducing substance even in the pres-

TABLE 3

*Influence of varying concentrations of choline and pantothenic acid on growth of Type II pneumococcus**

CHOLINE	NEPHELOMETER READINGS PANTOTHENIC ACID MICROGRAMS/ML.						
	5	2	1	0.5	0.25	0.1	0
micrograms/ml.							
50	2.0	2.1	2.4	>4.7	>4.7	—†	—
10	2.0	2.1	2.4	3.9	>4.7	—	—
2	2.3	2.4	2.4	2.5	2.6	4.2	—
0.4	3.9	4.1	4.3	4.6	>4.7	—	—
0.08	4.3	4.6	—	—	—	—	—
0.016	4.7	—	—	—	—	—	—
0	—	—	—	—	—	—	—

* Grown in basal medium (2). Constant in all tubes: Nicotinic acid 50 micrograms/ml.; thioglycollic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.

† No growth.

ence of optimum concentrations of the other essential factors is shown in table 4.

Flavin was not necessary for growth, but its addition increased the amount of growth slightly (table 5).

With the substitution of amino acids for gelatin hydrolysate it became possible to determine the growth values of particular amino acids. One amino acid at a time was withheld from the medium and the effect of its omission upon the growth of the different types observed (table 6). The question of the optimum concentration of each amino acid in the presence of the optimum concentrations of the other amino acids has not been investigated.

For the growth of Types II, V and VIII the amino acids were either indifferent, essential or inhibitory. Glutamic acid, leucine, arginine and histidine seemed to be essential for these three types.

TABLE 4

*Effect of thioglycollic acid**

THIOGLYCOLLIC ACID	NEPHELOMETER READING FOR TYPES			
	I	II	V	VIII
micrograms/ml.				
250	2.8	2.8	>4.7	>4.7
50	2.5	2.4	3.3	2.8
10	3.2	3.0	4.2	3.6
2	>4.7	>4.7	>4.7	>4.7
0.4	—†	—	—	—
0	—	—	—	—

* Type I grown in basal medium (1); Types II, V and VIII grown in basal medium (2). Constant in all tubes; Pantothenic acid 1 microgram/ml.; choline 2.5 micrograms/ml.; nicotinic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.

† No growth.

TABLE 5

*Effect of flavin**

FLAVIN	NEPHELOMETER READING FOR TYPES			
	I	II	V	VIII
micrograms/ml.				
1	2.7	2.7	3.7	3.1
0.5	2.5	2.6	3.5	3.0
0.25	2.5	2.4	3.4	2.9
0.1	2.4	2.4	3.3	2.8
0.05	2.6	2.6	3.5	2.9
0.025	2.7	2.6	3.6	2.9
0.01	2.7	2.7	3.6	3.0
0	2.7	2.7	3.7	3.1

* Type I grown in basal medium (1); Types II, V and VIII grown in basal medium (2). Constant in all tubes: Pantothenic acid 1 microgram/ml.; choline 2.5 micrograms/ml.; thioglycollic acid 50 micrograms/ml.

Methionine, on the other hand, was found to be non-essential for Type II, necessary to Type V, and perhaps slightly inhibitory for Type VIII. Lysine, non-essential for Type II and inhibitory for Type V, was definitely essential for Type VIII.

The failure of Type I to grow in the amino acid medium may be due to a deficiency or to an inhibitory effect of one or more amino acids for this organism. The cultivation of this type was attempted in an amino acid medium containing substances like thiochrome, Vitamins B_1 and B_6, or the amino acids serine, threo-

TABLE 6

*Amino acids and growth**

AMINO ACID OMITTED FROM MIXTURE	CONCENTRATION	NEPHELOMETER READINGS FOR TYPES:			
		I	II	V	VIII
	mgm./ml.				
d-Glutamic acid	1	—†	—	—	—
Glycine	0.25	>4.7	2.3	>4.7	3.5
l-Asparagin	0.2	>4.7	2.8	>4.7	>4.7
d-l Leucine	0.15	>4.7	>4.7	>4.7	>4.7
d-Arginine carbonate	0.075	—	—	—	—
d-l Alanine	0.05	>4.7	2.3	3.3	2.6
d-l Lysine dihydrochloride	0.05	>4.7	2.3	2.9	>4.7
d-l Methionine	0.05	>4.7	2.3	4.7	2.5
l-Cystine	0.05	>4.7	2.3	3.8	>4.7
d-l Histidine	0.025	—	—	—	—
l-Tryptophane	0.025	>4.7	2.8	3.2	2.7
β-alanine	0.025	>4.7	2.4	2.9	—
Nor-leucine	0.015	>4.7	2.3	2.9	2.5
l-Phenylalanine	0.01	>4.7	2.8	3.4	2.8
l-Oxyproline	0.01	>4.7	2.3	3.4	2.8
Control. None omitted	—	>4.7	2.3	3.4	2.8
Control. All omitted	—	—	—	—	—

* Constant in all tubes: Pantothenic acid 1 microgram/ml.; choline 2.5 micrograms/ml.; nicotinic acid 50 micrograms/ml.; thioglycollic acid 50 micrograms/ml.; flavin 0.1 microgram/ml.

† No growth.

nine, valine, iso-leucine, proline and tyrosine but in every case without success.

Media found adequate for Types I, II, V and VIII were deficient for Type VII. The addition of other substances such as Vitamins B_1 and B_6, thiochrome, uracil, guanylic acid, adenylic acid, inosinic acid, carnitine, compounds related to nicotinic acid, cadaverine, spermin, putrescine, pimelic acid, quinic acid, divicine, ergothionine, xanthine, hypoxanthine and others also

failed to induce the growth of this type. One strain, obtained from Dr. M. Finland, grew scantily in the regular amino acid medium.

Preliminary experiments done with one strain each of other types of pneumococci[4] gave readings ranging from 3.0 to 4.1 for Types VI, IX, XII, XIV, XXI, XXIV, and XXX grown in the amino acid medium.

DISCUSSION AND SUMMARY

Highly virulent strains of Types I, II, V and VIII pneumococci have been grown in a medium consisting of gelatin hydrolysate, certain additional amino acids, inorganic salts, glucose, choline, nicotinic acid, pantothenic acid and thioglycollic acid. Though in general comparable, both qualitative and quantitative differences were encountered in the essential factors for each type.

A mixture of known amino acids may replace the gelatin hydrolysate in media for Types II, V and VIII but will not support the growth of Type I. However, each type exhibited distinct amino acid requirements; an amino acid necessary for the growth of one type sometimes proved to be inhibitory or indifferent for another.

No growth was observed with Type VII in either medium even with the inclusion of such other compounds as pyridine derivatives, purines, pyrimidines, Vitamins B_1 and B_6 and thiochrome.

The addition of flavin gave slightly increased growth but was not essential.

Possible differences of growth requirements of strains within a type were not studied.

Note: Williams and Major (1940) published their paper on the structure of pantothenic acid about the time our experiments were concluded. Since then a preparation of synthetic pantothenic acid prepared in our laboratory from β-alanine and the acid component reported by Williams and Major has been found to replace the natural compound in the cultivation of Type II, the

[4] We wish to thank Dr. Finland for these organisms.

only type tested at this point. It follows that Type II at least may be grown in a medium chemically defined except for possible impurities in natural amino acids.

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