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Approved by: Milan A. Logan

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THE NUTRITION OF A STRAIN OF PNEUMOCOCCUS WITH A DETAILED STUDY OF THE CHOLINE REQUIREMENT

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fulfillment of the requirements of the degree of

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by

Elizabeth A. Badger
B. S. John B. Stetson University 1938
M. S. University of Cincinnati 1939

Contents

Introduction

The interest in choline and its derivatives has grown in recent years because of the unique role as hormone and as essential dietary and lipotropic factor. That little or no quantitative data can be found on the free choline content of the various biological fluids is due to the fact that the tissues contain approximately 1 mg. per cent free choline to 40-325 mgs. per cent total choline,(1) and good quantitative methods are not available for the analysis of small quantities in tissues.

The chemical methods used for the determination of choline (2-12) are for the most part based on the formation of an insoluble precipitate with iodine, platinic chloride, mercuric chloride, or Reinecke's acid. The resulting precipitates are purified and weighed as such, checked by melting point determinations, analyzed for nitrogen, trimethylamine, chromium, iodide, mercury or estimated colorimetrically. In most of the methods the final determination is made not on the choline part of the molecule but upon the added reagent. In micro-determinations this leads to errors through comtamination with excess reagent or through loss of the material by numerous washings. Samples containing a minimum of 15 micrograms of choline can be determined by the most sensitive chemical methods.

Some biological methods for choline determination depend on the conversion of the choline to its much more physiologically active acetic ester, and the subsequent assay of the latter on the frog's rectus abdominis muscle or the longitudinal muscle of the leech by comparison with standard acethlcholine solutions.(13) More recently developed is a biological assay which consists in comparing the amount of protection afforded by a food material and by pure choline chloride in preventing kidney hemorrhages which occur in rats receiving a choline-deficient diet. (11)

The recent development of microbiological assay methods for the determination of vitamins in biological fluids and tissues has gained rapid approval. The microbiological assays, i.e., vitamin assays in which the test agent used is some microorganism, are not only extremely sensitive to minute amounts of test material but are accurate, rapid and inexpensive. The minimum concentration of the vitamins determined by these microgiological methods is as follows: biotin 0.micro microgram (14), folic acid 0.0001 microgram (15), inositol 0.1 microgram (16), nicotinic acid 0.001 microgram (17), pantothenic acid 0.0001 microgram (18) *p*-aminobenzoic acid 0.01 microgram (19), pyridoxine 0.0001 microgram (20), riboflavin 0.001 microgram (21) and thiamine 0.0001 microgram (22,23)

The role of choline as an essential dietary constituent for fowls and mammals has been demonstrated repeatedly in recent investigations and it has been included by some as a member of the vitamin-B complex. The possibility of developing a microbiological assay for choline seemed wotthy of investigation.

It was first necessary to find a microorganism which required choline for growth. A survey of the literature of the nutritional requirements of microorganisms revealed only one organism which had been shown to require choline. Rane and Subbarow (24) had reported that choline, pantothenic acid and nicotinic acid are essential for the growth of certain types of Pneumococcus.

In order to determine the limitations and advantages accruing to the use of the Pneumococcus as a test agent, it was necessary to explore the nutritional requirements of the particular strain chosen for the investigation. A Type III (CHA) Pneumococcus was found to require pantothenic acid, nicotinic acid, biotin, choline as well as amino acids from a casein hydrolysate, cystine, certain inorganic salts, dextrose and a reducing agent. Within certain concentrations the growth was proportional to the amount of each of the vitamins present. The addition of choline in quantities as little as 0.1 microgram per ml. produced measurable growth. This organism therefore offered possibilities for the assay of choline as well as pantothenic acid, nicotinic acid and biotin.

To determine the specificity of choline as a growth factor for this Pneumococcus, many compounds which embodied part of the structural configuration of the choline molecule were investigated. It was found that the growth activity was contained in the N-C-C-OH part of the molecule. Only one naturally occurring substance, ethanolamine, was found to stimulate growth in the absence of choline. Ten molecules of ethanolamine were required to produce growth equal to that obtained with one molecule of choline. In the presence of suboptimal quantities of choline, ethanolamine added in amounts equivalent to five times the choline, produced no additional growth. These findings indicate that the growth of this organism offers definite possibilities for the determination of micro-quantities of free choline in biological materials.

In addition to the possible use as an assay procedure the study of the choline derivatives also contributed to the knowledge of the metabolic processes of the Pneumococcus. In this organism it was shown that choline does not function as a source of labile methyl groups, nor as a precursor of acetylcholine, glycine, serine or ethylamine but is probably required to form phospholipids.

The nutritional study of this Type III (CHA) Pneumococcus resulted in the development of a medium of essentially known constituents which not only supported growth equal to that in veal phosphate broth, but also maintained the virulence and type specificity of the organism after repeated subcultures. This is the first report of the growth requirements of a Type III Pneumococcus.

History of the Cultivation of the Pneumococcus

The Pneumococcus was first cultured in egg albumin (Klebs 1875), veal and beef broth (Pasteur 1881), then broth with the addition of gelatin or agar (Friedlander 1883), and later of blood, (Nissen 1891 and Gilbert and Fournier 1896), serum or other body fluids. (25) Since that time not much change has been made in the fundamental constituents of the media used for the cultivation of Pneumococcus. Detailed studies of the effect of the concentration and constituents of the cultural medium on the growth, viability and virulence of the Pneumococcus have resulted in the accumulation of certain facts about the nutritional requirements of this highly parasitic organism.

The essential ingredients of pneumococcal culture media have been shown to be meat extractives prepared from fresh muscle tissue, peptone, sugar, mineral salts and a suitable concentration of hydrogen ions.

Fresh beef, veal or horse muscle, freed from fat furnished a better base than the commercial meat extracts.(26) The addition of peptone to meat infusion broth resulted in two effects - inhibition or acceleration of growth, depending upon the kind of peptone and the method of preparation of the broth. Both Wright (26) and Dubos '27) studied this bacteriostatic action of peptone and found it could be neutralized by the addition of reduced thio compounds or by the addition of the peptone to the broth before it was heated.

Wright (28) also determined the necessity of a small amount of NaCl. Concentrations of salt, whether as a chloride or phosphate higher than 0.1 M, prevented growth of Pneumococcus (Dernby and Avery (29). Robertson, Sia and Woo (30) showed that 0.1 per cent gelatin in an electrolyte solution shielded the Pneumococcus from the toxic action of the electrolytes. Experiments of Hewitt (31) have shown that the presence of inorganic phosphate accelerated growth and breakdown of glucose by Pneumococcus, but his experiments were carried out in infusion broth sterilized by filtration rather than autoclaving; so as he indicates, the action may be different upon heating.

The addition of glucose to the medium stimulated and enhanced initial growth but because of the resulting acid production, resulted in eventual retardation or complete inhibition of growth. (Avery and Cullen(32) The acid formed could be neutralized either by the addition of sterile, powdered calcium carbonate (Wurtz and Mosny, and Hiss) or of small pieces of marble, washed and placed in the test tubes before filling and sterilization.(Bolduan)(25) That production of acid was also accompanied by a production of toxic peroxides was recognized by McLeod and Glovenlock.(33)

The initial hydrogen ion concentration of the medium and the changes occuring during growth were studied by Dernby and Avery.(29) They established the initial optimum pH at 7.8. Lord and Nye(34) have shown that as the medium becomes more acid (pH 7.4 to 6.8) the organism may live many days, between 6.8 to 5.1 death begins to take place; the greater the acidity the more rapid the death, while at 5.1 the cocci die within a few hours. Kelly(35) has shown that in the presence of animal fluids or their protein constituents the range in which the Pneumococcus will grow and survive is widened.

More nearly optimal growth conditions can be provided by the addition of a variety of substances. The favorable action of blood of man, rabbit, horse, or sheep was recognized by the earlier workers. In addition to contributing some nutritive substances, the blood acts as a buffer in controlling the reaction. Blood because of its oxidation-reduction system, tends to maintain a proper oxygen balance, while the iron in the hemoglobin seems to act as a catalase. Dubos(36) attempted to simulate the action of blood by the addition of certain iron compounds possessing catalase, peroxidase and oxidase activity in the presence or absence of yeast extract, but obtained favorable results only in prolonged viability of an avirulent Type II Pneumococcus.

Avery and Morgan(37) found that the addition to broth of sterile, unheated plant tissue, such as yellow and white turnip, carrot, beet, parsnip, white and sweet potatoe and green banana, not only caused acceleration of pneumococcal growth, but induced growth even when the inoculum was too small to initiate growth in the medium. The lag period was abolished and the stationary period extended and cell death delayed. The prolonged stationary period and delayed death was due to the presence of the enzymes catalase and peroxidase present in the vegetables which destroyed the peroxides formed by the Pneumococcus. Perhaps the acceleration effect was related to the production of carbon dioxide. Novy(38) has shown that raw potatoes when immersed in plain broth absorbs oxygen and produces carbon dioxide and the RQ may rise as high as 20. Kemper and Schlayer(39) have shown recently that the growth rate of Types I,II and III Pneumococcus is markedly dependent upon variations in carbon dioxide concentrations. This carbondioxide effect on the growth rate of Pneumococcus could be demonstrated even though the culture media contained peptone, blood and glucose.

Considering the complex nature of the pneumococcal cultivation, the development of a synthetic medium appeared all the more difficult. But with the progress of bacterial nutrition and the isolation of certain growth factors came the defined growth requirements of the Pneumococcus by Rane and Subbarow.(24) They obtained growth of highly virulent strains of Type I,II,V and VIII on a medium consisting of gelatin hydrolysate, certain additional amino acids, inorganic salts, glucose, choline, nicotinic acid, pantothenic acid and thioglycolic acid. A mixture of known amino acids could replace the gelatin hydrolysate for Type II,V and VIII. Rane(40) has since found biotin to be a growth essential for all Pneumococci tested. This biotin requirement was also confirmed by Landy et al.(41) with the use of avidin.

That the pneumococcal nutrition is far from being a complete story has been indicated by the difference encountered in the essential factors for each type. This variation of growth requirements of the different types and strains of Pneumococcus has also been noted by Anderson.(42)

Materials and Methods

In the study of bacterial nutrition the possibility of inhibition or stimulation of growth by traces of impurities in the chemicals or glassware must always be considered. To minimize this danger all glassware used in the preparation of the medium and in the growth tests was reserved for this purpose only and was kept scrupulously clean. Chemicals of the highest purity were used whereever possible. A list of the checmicals and their sources is given below.

Ascorbic acid (SMACO) Weighed amounts were added to medium just before autoclaving

Biotin (1) Lederle Biotin Concentrate contained 21 micrograms biotin per ml. 0.1 ml. of this concentrate was diluted to 50 ml. with distilled water and stored under toluene in the refrigerator.

(2) SMACO Biotin Concentrate #5000 contained 200 micrograms biotin per ml. 0.05 ml. was diluted to 50 ml. with distilled water and stored in the refrigerator.

(3) Crystalline Biotin (SMACO) Contents of a sterile ampoule containing 25 micrograms were transferred quantitatively to 50 ml. with distilled water and autoclaved 15 minutes at 10 lbs. pressure before storing in the refrigerator.

Calcium Pantothenate (SMACO) A stock solution was prepared containing 100 micrograms of calcium pantothenate per ml. dissolved in distilled water and stored in the refrigerator.

Choline Chloride (SMACO) A stock solution was prepared containing 1 mg. of choline chloride per ml. dissolved in distilled water and stored in the refrigerator.

Creatine (Coleman and Bell) A stock solution was prepared containing 1 mg. creatine per ml. dissolved in distilled water and stored in the refrigerator.

1 Cystine (SMACO) Added to basal medium in weighed amounts.

Glucose (Mallinckrodt Anhydrous Dextrose) 2.5 gms. were diluted to 10 ml. with distilled water and autoclaved for 15 minutes at 10 lbs. pressure.

L (+) Glutamic Acid (Amino Acid Mfg.) Added to basal medium in weighed amounts.

Glutamine (SMACO) Specific quantities weighed and added to the medium just before autoclaving. Due to a limited supply of this compound it was omitted from the medium used in the study of the choline derivatives.

Nicotinic Acid (Eastman) A stock solution was prepared containing 1 mg. of nicotinic acid per ml. dissolved in distilled water and stored in the refrigerator.

Thiamine Hydrochloride (SMACO) A stock solution was prepared containing 100 micrograms of thiamine hydrochloride per ml. dissolved in distilled water plus one drop of HCl and stored in the refrigerator.

<u>Uracil</u> (Eastman) A stock solution was prepared containing 1 mg. uracil.per ml. dissolved in a few drops of HCl by heating, diluted with distilled water and stored in the refrigerator.

The amines and choline derivatives were gathered together from various sources. The calcium salt of phosphorylcholine chloride was kindly supplied by Dr. Halvar Christensen. The diethylmethyl-β,γ -dihydroxypropylammonium chloride, α, α-dimethylcholine chloride, triethylcholine chloride and dimethylethylhydroxyethylammonium chloride were kindly supplied by Dr. Vincent du Vigneaud. The tetraethanolammoniumhydroxide, 2-amino-2-ethyl-1,3-propanediol, phenylethanolamine, diethylaminoethanol, dimethylethanolamine, methyldiethanolamine, and diethanolamine were generously contributed by the Carbide and Carbon Chemicals Co. I wish to express my thanks for these compounds. The remaining compounds were obtained from the following companies: Ethanolamine, N-acetylethanolamine, β-methoxyethylamine, triethanolamine, and -diethylaminopropanol from the Eastman Kodak Co. 2-Amino-2-methyl-1,3-propanediol, 2-amino-2-methylol-1,3-propanediol, and 2-amino-2-methyl-1-propanol from the Commercial Solvents Corporation. Acetylcholine chloride, acetyl-β-methylcholine chloride, carbamylcholine chloride, urethane of β-methylcholine chloride, and glycine from the Merck Chemical Company. Sarcosine (Pfanstiehl) methionine (SMACO) betaine (Hoffman la Roche) and dl-serine (Amino Acid Mfg.)

Solutions of the amines and choline derivatives were made by dissolving a weighed amount in distilled water.

<u>Measurement of Growth</u>

Turbidity - A turbidity comparator described by Krebs et al. (43) was used throughout this work. With this instrument light scattered by the particles passes to a photoelectric cell and the current from the photoelectric cell is amplified and read on a microammeter; thus the greater the turbidity, the larger the reading in microamperes. Tubes were chosen which gave a reading within 3.0 microamperes with a 0.2 per cent bentonite suspension. The growth tests were run in these matched tubes so that the turbidity could be measured directly at any time during the growth of the culture. Before the growth tubes were read, the instrument was set at 10 microamperes with a matched tube containing distilled water.

Acid Titration - The acid produced in a particular culture was measured by adding 0.1 N NaOH from a 5 ml. burette until the culture was just faintly alkaline to phenolpthalein. A small glass stirring rod with one end flattened was used to facilitate thorough mixing.

Bacterial Nitrogen - Bacterial nitrogen provides one of the most accurate methods for measurement of growth (44), but in this preliminary nutritional study a more rapid method of comparing growth was more practical. In a few experiments the turbidity measurements were checked with nitrogen determinations. Since the method used was a combination of several different methods (45, 46), the details are reported here. The 10 ml. culture was poured into a pyrex centrifuge tube and centrifuged at high speed (8000 R.P.M.) until the supernatant liquid was clear. This took about 30 minutes. As much of the supernatant liquid as possible was siphoned off with a capillary, the sediment washed with 5 ml. of distilled water and centrifuged a second time until the supernatant liquid was clear. The clear liquid was again siphoned off. Concentrated sulfuric acid (1 ml.) was added directly to the centrifuge tubes and the resulting solution transferred quantitatively with at least 6 one ml. washings to a 100 ml. Kjeldahl digestion flask. After the addition of 0.5 gm. K_2SO_4, two drops of a 5 per cent

$CuSO_4$ solution and a quartz pebble, the mixture was digested over a micro burner for ten minutes after the mixture became clear. The digested mixture was cooled and diluted to about 50 ml. with distilled water. A pinch of talcum, a drop of phenolpthalein and 4 ml. of a saturated solution of NaOH were added. The flask was immediately attached to a condenser and the NH_3 distilled directly into a flask containing 5 ml. of a 5 per cent solution of boric acid, 2 drops of methyl red and enough water to cover the end of the condenser. The boric acid solution was titrated with 0.01 N HCl to match a blank containing the same quantities of boric acid, indicator and water (46). Reagent blanks were run with each set of determinations. Young cultures were quite difficult to centrifuge clear and the results were quite inconsistent; but the values obtained on duplicate tubes of older cultures checked exceedingly well.

Plate counts - The number of viable organisms in a given culture was determined by making serial dilutions and plating. Serial dilutions were made by successive transfers of 1 ml. of the culture in 9 ml. portions of sterile basal medium. A fresh sterile Kahn pipette was used for each transfer and was rinsed 10 times before and after the transfer was made. Inoculations from these dilutions into the medium were always made in 0.1 ml. quantities. Plate counts were made by transferring 1 ml. of the 10^{-5} to 10^{-9} dilutions to sterile petri plates. The synthetic medium containing 1.2 per cent agar and cooled to about 45°C. was poured over the inoculum and mixed. Four plates for each dilution were made. The colonies on the plates were counted at the end of 18 and 24 hours incubation. The number of viable organisms present in the culture was estimated from the dilutions and the average number of colonies counted.

Bacteriological Methods

Cultures - Strains of Type I and Type II Pneumococcus were kindly supplied by Dr. Leo Rane at the Lederle Laboratories. The Type III Pneumococcus obtained from Dr. L. H. Schmidt at the Christ Hospital Institute for Medical Research was a strain known as Type III CHA. The stock cultures were passed through mice three times a week and the heart blood subcultured in a veal phosphate infusion broth, prepared according to the method in Park and Williams (47) for beef heart phosphate broth but with two exceptions. Lean veal was used in place of the beef heart and Difco proteose peptone in place of Parke-Davis peptone. The purity of the cultures was checked frequently by stained smears and the type specificity by the Neufeld reaction.

Inocula. To provide the inoculum for an experiment, 0.2 ml. of the stock culture was subcultured in 10 ml. of veal phosphate broth enriched with 0.25 per cent glucose and incubated for 5-6 hours at 34°C.* Five ml. of this young rapidly growing culture were transferred to a sterile centrifuge tube and centrifuged for 15 minutes. The supernatant liquid was pipetted off and the cells washed with 5 ml. of sterile basal medium. After centrifuging again for 15 minutes, the supernatant liquid was removed and the cells resuspended in 5 ml. sterile basal medium. The experimental tubes

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* Incubation at 34°C. was not chosen because it was optimum for the growth of this strain but because it was more convenient. The rate of growth at this temperature and at 37°C. did not differ greatly. The turbidimeter readings differed by 5 after 11 hours, by 13 after 16 hours, and by 0 after 18 hours incubation.

were immediately inoculated with 0.1 ml. of this suspension. The inoculations were made with a 1 ml. pipette and care was taken to prevent the contact of the pipette with the medium. This inoculum averaged around one million organisms per ml. of medium. This large inoculum was used for several reasons. (1) The development of growth was quite slow in the semisynthetic media and much time was saved by using an inoculum which would give rise to an earlier development of visible turbidity. (2) The results were more consistent. (3) The turbidity obtained without added choline was very slight. Experiments with smaller inocula have been reported in the section on bacteriological studies. A control tube of veal phosphate broth enriched with 0.25 per cent glucose was run with each experiment to check the inoculum and to serve also as a comparative standard of growth.

Chemical Methods

Preparation of Hydrolysates:

A modification of the method of Pappenheimer and Johnson (48) for the preparation of a gelatin hydrolysate has been used successfully in this work. 500 gms. of Eastman deashed gelatin or SMACO vitamin free casein was added slowly to 2500 ml. of 8 N HCl and hydrolyzed 18 hours over a flame. The dark brown hydrolysate was evaporated in vacuo to a gummy mass and redissolved in two liters of hot distilled water. This was again reduced to a gummy mass under reduced pressure. The black syrup was again dissolved in about 2 liters of hot distilled water and decolorized with Darco vegetable charcoal. This was accomplished by adding about 50 gms. charcoal to the hydrolysate and heating on a steam bath for about ten minutes. After standing about an hour the mixture was filtered by suction and if the filtrate was not a clear straw colored liquid more charcoal was added. During the course of this work, casein hydrolysates were prepared by this method three different times and there was no observable difference in their growth promoting qualitites. The nitrogen and chloride content of each preparation was determined. The nitrogen content of these hydrolysates, as determined by direct Nesslerization (49) after digestion with the Folin Kjeldahl digestion mixture (50), ran about 15-20 mgs.

percent N per ml. of hydrolysate. The chloride content, as determined by the Volhard titration method (51), ran about 80-100 mgs. NaCl per ml. of hydrolysate.

The hydrolysates were stored under toluene in the refrigerator.

A casein hydrolysate by both Pappenheimer's and Mueller's (52) methods with the removal of iron from portions of each is dexcribed below.

About 400 gms. of SMACO vitamine free casein was hydrolysed over a flame for 20 hrs. with 8 N HCl. The hydrolysate was evaporated to a gummy mass in vacuo and redissolved in about two liters of hot distilled water. Half of this mixture was evaporated again and decolorized with charcoal as described above. The other half was made up to two liters with distilled water and a paste of litharge (PbO) added until the hydrolysate was neutral to bromcresol green (pH 4). This suspension was then filtered by suction and the filtrate treated with about 50 ml. of a saturated solution of BaS (pure-yellow).

A solution of 10 N H_2SO_4 was added until the clear supernatant gave a ~~slight~~ precipitate with barium acetate. The addition of the BaS and H_2SO_4 was adjusted so that there was an excess of sulfide and a slight excess of sulfate. This suspension was filtered and the filtrate aerated for about two hours to remove excess H_2S. Iron was removed from 100 ml. portions of the two hydrolysates by absorption on $(Ca)_3(PO_4)_2$, One gram of KH_2PO_4 was added, the pH of the hydrolysate adjusted to 7.6-7.8, 2 ml. of a 10 per cent solution of $CaCl_2$ then added and the resulting solution heated to boiling and filtered while hot. The precipitation of $(Ca)_3(PO_4)_2$ was repeated three times.

Determination of pH of the basal medium was made first with phenol red, and then checked electrometrically. With practice it was possible to adjust the pH within 0.2 using the phenol red.

Procedure for Growth Tests.

Different amounts of the substance to be tested were added to matched pyrex tubes in duplicate concentrations. The growth factors were added from stock solutions to a calculated portion of the basal medium and the weighed amounts of ascorbic acid and glutamine added. Aliquote portions of this mixture were added to the tubes and thoroughly mixed. The total volume of each tube was made up to 9.7 ml. with distilled water, the tubes plugged with non-absorbent cotton and autoclaved for 15 minutes at 10 lbs. pressure. They were cooled slightly in water and while the sterile glucose solution was being added the inoculum was centrifuged the first time. While the inoculum was being centrifuged the second time the tubes were thoroughly shaken to insure complete mixing of the glucose. The inoculum was added and the tubes incubated. After 12, 18 and 24 hrs. incubation the tubes were removed from the incubator, shaken and the turbidities compared.

In the experimental data presented the turbidimeter values are the average values of duplicate tubes. Values differing by four microamperes or less are within experimental error.

Experimental Observations

Development of the medium.

Several attempts were made to grow strains of Type I and Type II Pneumococcus on the medium described by Rane and Subbarow (24). The medium was prepared as follows:

Medium I

Basal Medium

Acid-hydrolyzed Eastman de-ashed gelatin*......	18 ml. (=1 gm. N)
1 (+) Glutamic acid.........	0.1 Grams
1 Cystine....................	0.025 "
KH_2PO_4......................	5.0 "
Distilled water to make.....	800 ml.

pH adjusted to 7.8

To 8 ml. portions of the basal medium were added the following growth factors.

Calcium pantothenate	1 micrograms/ml. medium		
Nicotinic acid.............	10 "	"	"
Choline chloride...........	5 "	"	"

After autoclaving, the sterile solutions listed below were added.

$MgSO_4.7H_2O$..................	1.0 mgs./ml. medium		
Glucose....................	5.0 "	"	"
Riboflavin.................	0.1 micrograms/ml.medium		
Sodium Thioglycolate........ (10% soln.autoclaved)	50 "	"	"

* Hydrolysate prepared according to a modification of Pappenheimer's method.

The inoculum at this time was prepared as follows: Cultures of Type I and II Pneumococci obtained directly from the mouse were subcultured in a meat infusion peptone broth containing 0.1 per cent glucose, and incubated for 12 hours. 0.1 ml. of this culture was subcultured in the meat infusion broth with 0.1 per cent glucose and incubated 6-7 hours at 37°C., centrifuged, washed with sterile distilled water and resuspended in sterile distilled water. 0.1 ml. of this suspension was used to inoculate 10 ml. of medium.

With this medium and inoculum, the turbidity at the end of 60 hrs. incubation was very slight. The addition of biotin concentrate or the sterilization of the thioglycolate through a Seitz filter did not improve the growth.

Growth of the same organisms was next tried on a medium suggested by Dr. A. A. Anderson (42) and prepared as follows.

Medium II

$MgSO_4.7H_2O$	0.4	gms.
$CaCl_2.2H_2O$	0.02	"
$MnSO_4.4H_2O$	0.005	"
$FeSO_4.7H_2O$	0.005	"
Biotin (methyl ester).......	2.5	micrograms
Ca pantothenate...........	400.0	"
Choline chloride..........	10.0	mgs.
d (+) Glutamic acid.......	20.0	"
Glucose	4.0	gms.
K_2HPO_4	1.0	"
Distilled water to make ...	800	ml.

The pH was adjusted to 7.8 and the mixture heated and filtered. After autoclaving, 0.1 ml. each of sterile solutions of Coenzyme I* and thioglycolic acid ** were added to 8 ml. portions of the above medium.

The inoculum was the same as that used with Medium I; but there was still no turbidity at the end of 42 hours. Failure to obtain growth at this time was probably due more to inexperience in handling this organism than to the media.

The CHA strain of a Type III Pneumococcus was then tried in both medium I and II and in a combination of the two media. Measurable turbidity was obtained at the end of 42 hours with this organism in a medium prepared according to Rane but supplemented with biotin concentrate and the thioglycolic acid prepared according to Anderson's method.

* Coenzyme I solution: 5.0 mgs. Coenzyme I (50% pure) was dissolved in 25 ml. of distilled water. This solution was filtered through a Seitz filter and 2 ml. added to 8 ml. of sterile distilled water. 0.1 ml. of this dilution was added to 10 ml. medium.

** Thioglycolic acid solution was prepared by adding 0.2 ml. concd. thioglycolic acid to 100 ml. sterile distilled water.

The constituents per ml. of medium are shown in Medium III.

Medium III

Acid Hydrolysate of Eastman de-ashed gelatin......	1 mg. N
d (+) glutamic acid.............	100 micrograms
1 Cystine........................	25 micrograms
KH_2PO_4.........................	5 mgs.
Ca pantothenate..................	1 microgram
Nicotinic acid...................	10 micrograms
Biotin concentrate (Lederle).....	0.002 microgram
Choline chloride.................	5 micrograms
Added after autoclaving:	
Glucose..........................	5 mgs.
$MgSO_4.7H_2O$.....................	1 mg.
Thioglycolic acid................	8 micrograms
Riboflavin.......................	0.1 micrograms

The inoculum was also prepared in a different way. The stock culture was carried on veal phosphate infusion broth without any enrichment. For the inoculum 0.2 ml. of the stock culture was subcultured in veal phosphate medium enriched with 0.25 per cent glucose and incubated 6 hours. Five mls. of this subculture were centrifuged, washed and resuspended in the same volume of sterile basal medium. 0.1 ml. of this suspension was used to inoculate 10 ml. of medium.

With Medium III and this inoculum a turbidimeter reading of around 65 was obtained after 48 hours incubation with choline and a reading of 10 (the same as that given with distilled water) without choline.

Growth in this semi-synthetic medium was far inferior to growth in the veal phosphate infusion broth. Consequently, a detailed qualitative and quantitative study of the constituents of this medium was undertaken.

Qualitative studies of Medium III.

The protein hydrolysate was the first constituent subjected to analysis. In these experiments the methods were essentially the same. A basal mixture consisting of

l cystine...............	25 mgs.
d (+) glutamic acid......	100 mgs.
KH_2PO_4..................	5 gms.
Distd. water to make....	800 ml.
pH adjusted to 7.8	

was prepared and used over a period of four to six days. The protein hydrolysates were added to small portions of this basal mixture just before testing and the pH readjusted to 7.8. The growth factors were added before autoclaving in the same concentrations as Medium III and the glucose, $MgSO_4$, thioglycolic acid and riboflavin added after autoclaving.

A vitamin free casein hydrolysate was prepared in the same way as the gelatin hydrolysate (Pappenheimer's method) and the growth obtained with these two hydrolysates was compared. Table 1.

Table 1

Comparison of Growth Obtained with Gelatin and Casein Acid Hydrolysates.

	Turbidimeter Readings	
	20 hrs.	43 hrs.
Gelatin Hydrolysate + choline	30	52
" " - choline	10	15
Casein Hydrolysate + choline	41	100
" " - choline	12	17

The vitamin free casein hydrolysate supported better growth than the gelatin and gave as low a blank without added choline.

As has been described above the mixture of cystine, KH_2PO_4 and glutamic acid was sufficient for several experiments and was kept as long as a week in the refrigerator. A comparison of the results of many experiments indicated that the most consistently rapid growth was obtained with a basal medium in which the hydrolysate had been added to the basal mixture on the same day that the latter was prepared. Typical results are given in Table 2.

Table 2

Effect of Freshly Prepared Basal Mixture in Decreasing the Lag Phase

Date Basal Mixture Prepared	Date Hydrolysate added to Basal Mixture	Date of test	Turbidimeter Readings	
			16 hrs.	24 hrs.
7-23	7-23	7-23	35	48
7-20	7-20	7-23	19	39

Another time factor was involved in the observation that the lag period was shortened when the casein hydrolysate and the basal mixture were combined at least twenty-four hours before the growth test was run. This ageing effect is illustrated in Table 3.

Table 3

The Effect on Initiation of Growth of Aged Basal Medium

	Turbidimeter Readings	
	16 hrs.	40 hrs.
Basal Medium prepared and tested the same day	10	40
The same basal medium tested the following day	74	117

This acceleration of growth with the aging of the basal medium seemed to suggest the neutralization or antagonism of some inhibitory constituent of the hydrolysate by the cystine, glutamic acid, KH_2PO_4 or the slightly alkaline pH. To investigate this latter possibility a part of the acid casein hydrolysate was neutralized to pH 7.6-7.8 with NaOH and allowed to stand 24 hrs. before adding the cystine, glutamic acid and phosphate. At the same time the acid hydrolysate was combined with the three other constituents of the basal medium, the pH adjusted to 7.8 and the resulting solution was allowed to stand 24 hrs. before testing. Table 4 compares the growth produced by these two different basal media.

Table 4

Effect of Neutralizing the Acid Hydrolysate

	Turbidimeter Readings	
	15 hrs.	27 hrs.
Acid Hyd. + basal mixture 24 hrs. before testing	50	76
Neutralized Hyd. + basal mixture 1 hr. before testing	10	20

That the pH was not the contributing factor in this aging process was also brought out in later experiments with cystine. In these experiments the basal medium contained the casein hydrolysate, glutamic acid and phosphate but no cystine. The rate and extent of growth was the same on the day the basal medium was prepared as it was four days after.

The effect of cystine on the casein hydrolysate or perhaps vice versa was shown in this same experiment and is illustrated in Table 5.

Table 5

Effect of Cystine on Casein Hydrolysate

	Turbidimeter Readings 12 hrs.	18 hrs.
1 - Basal Medium with cystine prepared 9 days before testing	69	103
2 - Basal Medium without cystine prepared 4 days before testing	47	66
Cystine added to basal Medium (2) 1 hr. before testing	56	90
Cystine added to basal Medium (2) 24 hrs. before testing	63	95

Although the difference is not great it is seen that a more rapid growth was obtained when the cystine and hydrolysate were in solution together at least 24 hrs.

The possibility of obtaining a hydrolysate which would not require this aging led to experiments with different preparations of casein hydrolysate. In this laboratory a casein hydrolysate prepared according to Mueller's method had been used successfully in promoting growth of Cl. Welchii (53). This differed from Pappenheimer's method in that the excess chloride was removed with PbO, the Pb subsequently removed with BaS and the excess with H_2SO_4.

Half of a vitamin free casein hydrochloric acid hydrolysate was prepared according to Pappenheimer's method and half according to Mueller's method. In view of the recognized importance of iron in bacterial nutrition (54), this element was removed from portions of the two hydrolysates by repeated treatments with KH_2PO_4 and $CaCl_2$.

Basal medium was prepared with each one of these hydrolysate preparations in a quantity sufficient to provide 0.5 mg. N per ml. medium. At the same time a basal medium containing Casamino Acids (Difco) was prepared. The media were tested the same day that they were prepared and again three days later. The growth produced by these hydrolysates is compared in Table 6.

Table 6

Comparison of Casein Hydrolysate Preparations

	Turbidimeter Readings			
	First Day		Three Days Later	
	12 hrs.	18 hrs.	12 hrs.	18 hrs.
Charcoal treated acid hyd. + choline	57	83	60	94
Charcoal treated acid hyd. - choline	13	17	14	17
Charcoal treated neutral hyd. - iron	53	72	57	91
Lead treated hydrolysate	52	72	55	85
Lead treated hyd. - iron	53	68	58	92
Casamino acids + choline	54	80		
Casamino acids - choline	32	32		

It is seen from Table 6 that the removal of iron or excess chloride did not effect the aging process or improve the rate or extent of growth. The casamino acids provided no faster growth and gave a high blank without added choline. No improvement was found over the charcoal treated hydrolysate. Experiments with a SMACO casein hydrolysate also gave improved growth on standing. Other experiments in which the basal medium was treated with KH_2PO_4 and $CaCl_2$ to remove iron, failed to show any change in rate or extent of growth. Further study of the aging process must wait until the amino acid requirements of this organism are determined.

Experiments with the $MgSO_4$indicated that the concentration used (1 mg. per ml. medium) was too high: See Table 7.

Table 7

Effect of $MgSO_4$ Concentration

$MgSO_4$ added to tubes after autoclaving	Turbidimeter Readings 45 hrs.	 62 hrs.
1 mg. per ml. medium	10	10
0.5 mg. per ml. medium	33	50

By lowering the concentration to 0.5 mg. $MgSO_4.7H_2O$ per ml. medium, it was possible to add the $MgSO_4$ to the basal medium and still retain a clear medium after autoclaving.

Table 8

Effect of the Addition of $MgSO_4$ to Medium Before Autoclaving.

	Turbidimeter Readings 18 hrs.	 26 hrs.
0.5 mg. $MgSO_4$ added before autoclaving	53	77
0.5 mg. $MgSO_4$ added after autoclaving	45	75

These changes in the preparation of the basal medium may be summarized in the following description. The cystine (25 mg.) was dissolved in the acid vitamin-free casein hydrolysate (700 mg. N) by heating gently. About 500 ml. of distilled water was added to this mixture before adding the glutamic acid (100 mg.), KH_2PO_4(5 gms.) and $MgSO_4$ (0.5gm.). The volume was made up to 800 ml. with distilled water and the pH adjusted to 7.8 with concentrated NaOH. The neutralized solution was heated to boiling and filtered through filter paper while hot. This basal medium was prepared at least two days before using.

These changes in the preparation of the basal medium increased the growth and shortened the lag period; but the addition of riboflavin, thioglycollic acid and glucose after autoclaving was much too cumbersome for assay purposes.

The addition of Riboflavin did not have any apparent effect on the rate or extent of growth in the concentrations tried, so it was dropped from the medium. The riboflavin solution in 0.02 N acetic acid was autoclaved separately and added to the sterile medium.

Table 9

The Effect of Riboflavin

Riboflavin microgram/ml. medium	Turbidimeter Readings 12 hrs.	18 hrs.
0	74	122
0.1	73	119
1	74	118
5	73	109

Dr. Rane (40) had found it possible to replace thioglycolic acid with ascorbic acid (100 micrograms per ml. medium) and glutathione (0.1 microgram per ml.) adding both to the medium before autoclaving. The results in Table 10 confirm this finding.

Table 10

Effect of Replacing Thioglycolic Acid by Ascorbic Acid and Glutathione

	Turbidimeter Readings.	
	17 hrs.	24 hrs.
Thioglycolic acid added after autoclaving.	21	41
Ascorbic acid and glutathione added before autoclaving	24	48

Other experiments showed that the glutathione was not necessary.

Table 11

Effect of Glutathione in the Presence of Ascorbic Acid.

Glutathione µmgs./ml. medium	Ascorbic acid µmgs./ml. medium	Turbidimeter Readings 18 hrs.	26 hrs.
0.1	100	63	92
0.1	200	63	93
0.1	300	65	98
0	100	67	98
0	200	67	99
0	300	67	105

Since the addition of glutathione to the medium did not produce any apparent beneficial effect, it was not included.

The only substance remaining to be added after autoclaving was glucose. Repeated experiments indicated that growth in the medium to which the glucose was added before autoclaving did not equal that to which the glucose had been added after autoclaving.

Table 12

Effect of The Addition of Glucose Before and After Autoclaving.

	Turbidimeter Readings	
	12 hrs.	18 hrs.
5 mg./ ml. glucose added before autoclaving	63	89
5 mg./ ml. glucose added after autoclaving	67	106
5 mg./ ml. glucose added before and after autoclaving	61	87

Part of the glucose was probably altered when autoclaved at this slightly alkaline pH. That the lower growth may have been due to the formation of inhibitory products rather than to the reduction in the concentration of utilizable glucose was indicated by the lower growth in the tubes which received glucose before and after autoclaving. For assay purposes, the addition of glucose to the medium before autoclaving would be quite a time saver as well as insuring more uniform results. However, while the optimum concentrations and constituents of the medium were being worked out it was thought best to avoid adding any further unknown factors. Separate sterilization of the glucose was continued but instead of adding 0.1 ml. of a 50 per cent solution of glucose, 0.2 ml. of a 25 per cent solution was added to each tube. Even then extreme care was necessary to obtain a homogeneous mixture. The best results were obtained by rotating the tubes with a circular movement from the wrist.

These qualitative changes are incorporated in Medium IV.

<u>Medium IV</u>.

Basal Medium

Vitamin free casein hydrolysate	700 mgs. N
1 cystine	25 mgs.
1 (+) glutamic acid	100 mgs.
KH_2PO_4	5 gms.
$MgSO_4.7H_2O$	500 mgs.
Distilled water to make	800 ml.

pH adjusted to 7.8, heated to boiling, filtered and stored in refrigerator two days before using.

To 8 ml. portions of the basal medium were added the following substances:

Calcium pantothenate	1.0	micrograms/ml.		medium
Nicotinic acid	10.0	"	"	"
Biotin (concentrate)	0.002	"	"	"
Choline chloride	5.0	"	"	"
Ascorbic acid	300.	"	"	"
Glucose (after autoclaving)	5.0 mg./ml. medium			

With this medium growth approaching that in veal phosphate broth in both time and extent was obtained, but the lag period with the semi-synthetic medium was still three to four hours longer than in the veal phosphate broth. During the development of the medium many different substances were added in an effort to find some factors which would increase the rate of growth and decrease the lag period. Also for assay purposes it was necessary to incorporate as far as possible all growth stimulatory substances which might occur in the test materials.

In order to put the results of the study of these growth accelerating factors on a comparable basis, the ratio times 100 of the turbidity with and without the added substance has been computed. Therefore a value of 100 indicates no effect, a value greater than 100 indicates an accelerating effect and a value of less than 100 indicates an inhibitory effect. Values differing by ± 2 are within experimental error. Different experiments are separated by a space. All compounds were added to the medium before autoclaving unless otherwise indicated. See Table 13 for a summary of the effects of these compounds.

Table 13

The Effect of Known Growth Accelerator Substances

Name of Compound micrograms/ml. medium	Turbidimeter Readings 12 hrs.	Turbidimeter Readings 18 hrs.
Adenine Sulfate		
0.5	104	104
2.5	105	106
5.0	101	97
Adenylic acid		
0.2	86	103
1.0	99	101
2.0	96	99
Asparagin		
1.0	100	100
5.0	102	100
10.0	103	104
20.0	94	101
30.0	88	96
40.0	83	91
Beta-alanine		
0.5	100	107
1.0	99	105
0.5	99	104
1.0	99	99
5.0	97	97
Carnitine		
1.0		98
5.0		102
10.0		90
Creatine		
1.0		103
5.0		107
10.0	97	97
5.0	100	99
2.5	100	105
6.0	101	103
12.0	101	104
18.0	101	105
10.0 (C.B.)*	102	106
Folic acid**		
0.002	94	90
0.004	96	94
0.012	95	92
0.016	93	86
Glucosamine		
1.0		91
5.0		75
10.0		70
15.0		85
100		83
1.0	93	92
2.0	106	100
10.0	97	102
20.0	99	102
Glutamine		
250	118	121
500	118	124
50		110
100		112
150		112
200		114
250		115
300		107
350		116
400		105
450		110
500		120
75 (C.B.)	96	110
150 (C.B.)	101	112
150 (C.B.)[a]	103	114
225 (C.B.)	101	110
Glutathione		
0.1	100	101
0.5	91	86
1.0	86	74
0.5 (C.B.)	88	99

* (C.B.) growth test was run with crystalline biotin.

** Folic acid concentrate in 20% alcohol. Inhibition shown to be due to the alcohol.

[a] Added after autoclaving

Table 13 (Continued)

Name of Compound micrograms/ml. medium	Turbidimeter Readings	
	12 hrs.	18 hrs.
Guanosine		
0.2	85	103
1.0	90	95
2.0	108	98
Inosotol		
1.0		95
5.0		94
10.0		91
100		90
0.01	102	101
0.1	100	100
Methionine		
5.0		100
10.0		91
100		96
Nicotinamide		
5.0	99	98
10.0	101	100
20.0	100	98
Para-aminobenzoic acid		
1.0		95
5.0		98
10.0		94
100		95
0.01	96	100
0.1	102	99
Pimelic acid		
0.004	100	99
0.04	95	100
0.4	98	106
4.0	98	107
Phthiocol*		
1.0	86	100
5.0	57	105
10.0	23	30
0.4	95	97
1.2	97	97
2.0	101	101
3.0	97	98
1.0 (C.B.)	83	97
Pyridoxine		
2.5	99	102
5.0	99	102
25.0	97	101
Sarcosine		
1.0		103
5.0		104
10.0		96
Thiamine		
0.25	98	107
0.5	104	106
0.5 (A)	106	105
1.0	103	105
0.5	100	108
Tryptophane		
0.2	92	93
10.0	86	96
20.0	102	99

* Phthiocol (Endo Products) 2 mg. 2-methyl-1,4-naphthoquinone plus 2.66 mg. sodium bisulfite per ml. stock solution.

Table 13 (Continued)

Name of Compound micrograms/ml. medium	Turbidimeter Readings	
	12 hrs.	18 hrs.
Uracil		
2.0	102	110
5.0	102	107
10.0	101	104
20.0	101	103
50.0	101	103
5.0 (C.B.)	95	102
Urea		
0.5	98	101
1.0	97	97
5.0	97	94
10.0	99	99

There was a slight indication of acceleration of growth with creatine, glutamine, thiamine and uracil; so these substances were added to the medium. But in general the results presented in Table 13 showed that the addition of these compounds to this semi-synthetic medium in the concentrations given did not accelerate the growth or decrease the lag period to any measurable extent. Perhaps their effect was masked by some limiting factor of the medium. In the study of accessory growth substances, the conditions in which they produce a favorable action may be dependent not only upon the limited concentration of the substance itself but also upon the presence and quantity of the other medium constituents. Before any conclusions can be drawn about accelerating growth factors for this Pneumococcus, repeated tests must be made with a wider range of concentrations and with a medium in which the casein hydrolysate is replaced with amino acids.

Medium V

Medium IV plus creatine, glutamine, uracil and thiamine in the following concentrations

creatin	6	micrograms	/ml.	medium
glutamine	150	"	"	"
uracil	5	"	"	"
thiamine	0.5	"	"	"

has been designated as Medium V for the sake of reference. Bacterial and quantitative studies were conducted on this medium.

Quantitative Studies of Medium V

It seemed safe to conclude from the amount and rate of growth obtained with Medium V that all the essential growth factors were present. A preliminary determination of the optimum concentration of each constituent has been made as the medium was evolved. In view of the changes made, some substances added and some removed, a detailed quantitative study of the medium was in order.

Casein Hydrolysate.

To determine the optimum concentration of the casein hydrolysate varying amounts of the hydrolysate were added to a mixture of cystine, glutamic acid, KH_2PO_4 and $Mg\ SO_4$ in duplicate tubes, the volume made up to six ml., the pH adjusted to 7.8 and the tubes heated in the autoclave at five pounds pressure for five minutes. The tubes were then stored in the refrigerator for two days. At the end of this time the growth factors and ascorbic acid were added in the usual manner and the total volume made up to 9.7 ml. with distilled water before autoclaving. The results of the experiment are given in Table 14.

Table 14

Optimum Concentration of the Vitamin Free Casein Hydrolysate.

Mgs.N/ml. medium	Turbidimeter Readings 12 hrs.	18 hrs.
0.31	69	100
0.47	70	106
0.66	65	99
0.78	66	88
0.94	60	74
1.09	61	63

As seen from the results in Table 14 the concentration was not critical but there was a definite trend toward inhibition in the higher concentrations. Experiments with two different hydrolysate preparations indicated an optimum concentration around 0.5 mg. nitrogen per ml. medium. An assay carried out with a SMACO hydrolysate of vitamin free casein pointed to a slightly higher optimum concentration. See Table 15.

Table 15

Optimum Concentration of SMACO hydrolysate of Casein

Mgs. N/ml. medium	Turbidimeter Readings	
	12 hrs.	18 hrs.
0.1	57	78
0.2	56	76
0.3	58	82
0.4	62	87
0.5	62	89
0.6	65	88
0.7	60	97
0.8	60	97
1.0	58	97

In subsequent experiments the casein hydrolysate was present in a concentration of 0.5 mgs. nitrogen per ml. medium.

Cystine.

In the same experiment described above in which the hydrolysate was varied, tubes containing varying quantities of cystine were also set up. The resulting growth is shown in table 16.

Table 16

Effect of Cystine

Micrograms Cystine per ml. medium	Turbidimeter Readings	
	13 hrs.	18 hrs.
12	65	90
19	65	97
25	65	99
31	65	99

This relatively small difference in growth obtained over a wide range of cystine concentration was substantiated by results of another experiment in which the basal madium without cystine was prepared four days before the growth test was run. Various concentrations of cystine were added to portions of 8 ml. of the basal medium and the pH re-adjusted to 7.8 before the growth factors and ascorbic acid were added.

Table 17

Effect of Cystine

Micrograms Cystine per ml. medium	Turbidimeter Readings 12 hrs.	18 hrs.
0	53	71
10.0	54	74
15.0	61	87
20.0	63	93
25.0	61	90
30.0	60	90
40.0	59	86
50.0	57	78

From this experiment it is seen that growth was supported by this medium without the addition of cystine, but the rate and extent of growth was much improved by the addition of 20-25 micrograms cystine per ml. medium. The amount of cystine contributed by the casein hydrolysate, as calculated from the cystine content of casein (55), is about 2 micrograms per ml. medium. This does not include any lose of cystine in the process of making the casein vitamin free or in preparing the acid hydrolysate. Until the amino acid requirement of this organism is determined, one cannot place cystine among the essential substances required for growth.

In the study of the sulfur requirements of bacteria it has been found that cystine may function as a growth promoter for at least three reasons (56, 57): (1) as a non-specific source of organic sulfur, (2) as a source of the -S-S-linkage, (3) as an essential amino acid needed to form cell constituents. In animal diets methionine can replace cystine. By demethylation the methionine is converted to homocysteine which can in turn be oxidized to homocystine ans as such can replace cystine.

To test the specificity of cystine as a growth accelerator for this organism different sulfur compounds were tried, and the results presented in Table 18.

Table 18

Effect of sulfur Compounds Other than Cystine.

Sulfur Compound	Micrograms per ml. medium	Turbidimeter Readings 12 hrs.	18 hrs.	24 hrs.
Cystine	0	36	48	68
	20	57	72	113
Cysteine	10	49	56	110
	20	57	70	110
	30	57	71	114
	40	55	69	113
Methionine	15	36	43	64
	25	36	43	63
	50	37	44	65
Homocysteine	15	37	48	78
	25	38	47	77
	35	37	47	78
	50	38	47	78
Glutathione	30	56	67	113
	60	54	63	108
	90	54	60	105
	120	53	60	103
Thioglycolate	5	39	47	75
	10	37	49	75
	20	39	55	87

From the results in Table 18 it is seen that cysteine and glutathione can replace cystine but methionine, homocysteine and thioglycolate in the concentrations used cannot replace cystine. This suggests that cystine may function as an essential amino acid.

Glutamic acid

The effect of added glutamic acid was tried again and again. There appeared to be no difference in growth when it was added or left out of the medium. Table 19 shows the effect of adding different concentrations.

Table 19

Effect of Added Glutamic Acid

Glutamic Acid micrograms/ml. medium.	Turbidimeter Readings	
	11 hrs.	18 hrs.
0	67	112
25	68	112
50	68	111
75	68	113
100	67	109
125	64	108
150	65	107

The effect of added glutamic acid was also tested with crystalline biotin but there was still no positive evidence that it exerts a beneficial effect. The glutamic acid requirement is probably satisfied by the casein hydrolysate, since according to Mathews (56) 21.7 per cent of casein is glutamic acid in contrast to 5.8 per cent in gelatin.

Magnesium Sulfate

It has previously been shown that better growth resulted when the concentration of $MgSO_4.7H_2O$ was dropped from 1.0 mg. to 0.5 mg. per ml. of medium. A more detailed study of the magnesium sulfate requirement showed that it was essential for growth but the concentration required was not critical.

Table 20

Effect of Magnesium Sulfate

$MgSO_4.7H_2O$ mgs./ml. medium	Turbidimeter Readings 13 hrs.	19 hrs.
0	13	13
0.2	53	74
0.4	53	80
0.5	54	83
0.6	54	75
0.7	55	73

Potassium Acid Phosphate

The optimum concentration of potassium acid phosphate was studied and found to be the same as that used by Rane, 5 mg./ml. medium.

Table 21

Effect of Potassium Acid Phosphate

KH_2PO_4 mgs./ml. medium	Turbidimeter Readings 12 hrs.	19 hrs.
2.0	40	57
3.0	45	68
4.0	49	70
5.0	53	73
6.0	54	63
7.0	55	62

Experiments with sodium-β-glycerophosphate indicated that it could not replace KH_2PO_4 but could be substituted for part of the KH_2PO_4.

Table 22

Effect of Sodium-β-glycerophosphate

KH_2PO_4 Mgs./ml. medium	Sodium-β-glycerophosphate mgs./ml. medium	Turbidimeter Readings 12 hrs.	19 hrs.
5.0	0	53	73
0	5.0	35	45
2.0	1.0	42	62
3.0	0	45	68
2.0	2.0	48	73
4.0	0	49	70
2.0	3.0	48	71

Using 0.5 mg. $MgSO_4.7H_2O$ and 5.0 mg KH_2PO_4 per ml. of medium the total molar concentration of the inorganic salts including the NaCl of the hydrolysate was about 0.05, well within the limit of 0.1 molar determined by Dernby and Avery(29).

Traces of Cu, Zn, Th, Ca, and Mn added to the medium produced no change in rate of growth or length of lag period. The effect of the removal of iron has already been mentioned in the experiments with the casein hydrolysates. Undoubtedly there were traces of different metals present in the medium and a more detailed study of those metals and their concentrations might be profitable in shortening the lag period.

Hydrogen Ion Concentration of the Basal Medium.

Preliminary experiments as well as experience of other workers with more complex media indicated the optimum initial pH for Pneumococcus to be around 7.8 (29).

The pH of aliquot portions of the basal medium was adjusted at 0.2 intervals in a range of 6.8-8.0. The hydrogen ion concentration was measured electrometrically and triplicate tubes were run for each pH. The effect of the pH on rate and extent of growth is shown in Table 23.

Table 23

Effect of pH of the Basal Medium

pH of Basal Medium	Turbidimeter Readings		
	12 hrs.	18 hrs.	24 hrs.
6.8	35	54	76
7.0	44	76	89
7.2	50	84	104
7.4	55	84	112
7.6	62	88	127
7.8	61	84	128
8.0	57	72	126

A pH of 7.6-7.8 seems to be optimum for all periods at which the growth was measured. In view of the relative instability of most growth factors on the alkaline side of 7 and the fact that this pH optimum holds true for the more complex media, it would seem that the hydrogen ion concentration has a direct action on the initial growth of this organism rather than an indirect action through the constituents of the medium.

Ascorbic Acid

Table 24 shows the rather wide limits of ascorbic acid optimal concentrations.

Table 24

Effect of Ascorbic Acid

Ascorbic Acid Micrograms per ml. medium	Turbidimeter Readings 11 hrs.	18 hrs.
0	11	12
100	57	84
150	57	84
200	60	89
250	61	88
300	63	92
350	63	87
400	63	89

Several ascorbic acid titrations seemed to point to a possible optimum at 300 micrograms per ml. medium. The exact optimum oxidation potential for this medium has not yet been determined; but experiments by Dubos (36) indicate that pneumococci can multiply only in a medium in which the oxidation potential is below a critical level $E_o = -0.046$ v.

at a pH of 7.4. A study of the optimal oxidation reduction potential of this medium would probably aid in the reduction of the lag period.

Glucose

Avery and Cullen (32) found that more than 0.4% carbohydrate must be present to reach maximum acidity of pH 5.0. That this minimal carbohydrate concentration holds true for growth in the semi-synthetic medium is shown in Table 25. A 25% glucose solution was autoclaved 15 minutes at 10 lbs. pressure and added to the sterile medium in the concentrations noted. The effect of adding the glucose to the medium prior to sterilization has been discussed.

Table 25

Effect of Glucose

Glucose mgs./ml. medium	Turbidimeter Readings 12 hrs.	18 hrs.
0	12	12
0.6	16	17
1.2	25	28
3.1	49	87
3.7	55	93
4.3	62	98
5.0	54	98
6.2	58	95

Biotin

Earlier experiments had shown that a Lederle biotin concentrate containing 21 micrograms of biotin per ml. could be replaced by crystalline biotin. The concentrate was used because it was more economical and more readily available. When this supply of concentrate was exhausted a SMACO concentrate containing 200 micrograms biotin per ml. was substituted. To obtain equivalent growth four times as much SMACO biotin was required as Lederle biotin. When crystalline biotin was used to check the biotin content of the SMACO concentrate no growth resulted. After comparing the medium used in the earlier experiment with crystalline biotin it was found that nicotinic acid was the essential missing compound. While using the Lederle biotin concentrate the addition of nicotinic acid to the medium produced no change in growth so it was considered unessential and dropped from the medium. By adding it again to the medium growth was obtained with the crystalline biotin. That the concentrates also contained pantothenic acid was shown by a difference in growth without added pantothenic acid with the crystalline biotin used in place of the biotin concentrate. Table 26 gives some idea of the nicotinic and pantothenic acid content of the two biotin concentrates.

Table 26

Nicotinic Acid and Pantothenic Acid Content in Two Biotin Concentrates

Lederle Biotin Concentrate (21 micrograms biotin per ml. concentrate).

Biotin µmgs./ml. medium	Concentrate ml./ml. medium	Nicotinic A. µmgs.added/ ml.medium	Pantothenic A. µmgs. added/ ml.medium	Turbidimeter Readings	
				12 hrs.	18 hrs.
0.002	0.0001	0	1	65	105
0.002	0.0001	8	1	63	100
0.002	0.0001	16	1	66	103
0.002	0.0001	10	0	35	44
0.002	0.0001	10	0.03	63	109
SMACO biotin concentrate (200 micrograms biotin per ml. concentrate)				18 hrs.	41 hrs.
0.002	0.00001	0	1	56	116
0.002	0.00001	0	0	12	12
Crystalline biotin				12 hrs.	18 hrs.
0.001		0	1	14	16
0.001		0.2	1	71	111
0.001		1	0	13	13
0.001		1	0.2	74	114

By comparing growth curves obtained with the biotin concentrates and those obtained with varying concentrations of nicotinic acid and pantothenic acid using crystalline biotin, it was possible to estimate the nicotinic acid and pantothenic acid content of the concentrates. The Lederle biotin concentrate contained at least 2 mgs. nicotinic acid and about 200 micrograms of pantothenic acid per ml. concentrate.

The SMACO biotin concentrate contained about 2 mgs. nicotinic acid and less than 125 micrograms pantothenic acid per ml. concentrate.

It is not probable that the biotin concentrates contained any other growth factor essential for this organism since the same rate and extent of growth was obtained with the crystalline biotin as with the biotin concentrates when optimum concentrations of the growth factors were present. No appreciable amount of choline was present in the concentrate since the growth without choline was the same when either the crystalline biotin or biotin concentrate was used. In so far as time permitted experiments in which the biotin concentrate had been used were repeated with crystalline biotin. This experience with the biotin concentrate emphasized again the importance of using as far as possible the purest chemicals for nutritional studies.

The minimum effective concentration of biotin was determined and the results are given in Table 27 and Graph I.

Table 27

Effect of Biotin

Micrograms Biotin per ml. medium	Turbidimeter Readings	
	<u>12 hrs</u>.	<u>18 hrs</u>.
0	15	15
0.00002	27	30
0.00004	38	50
0.00008	54	84
0.0002	65	103
0.0004	70	116
0.0006	64	110
0.0008	68	114
0.001	70	115

Graph I

Effect of Biotin on the Growth of a Type III Pneumococcus

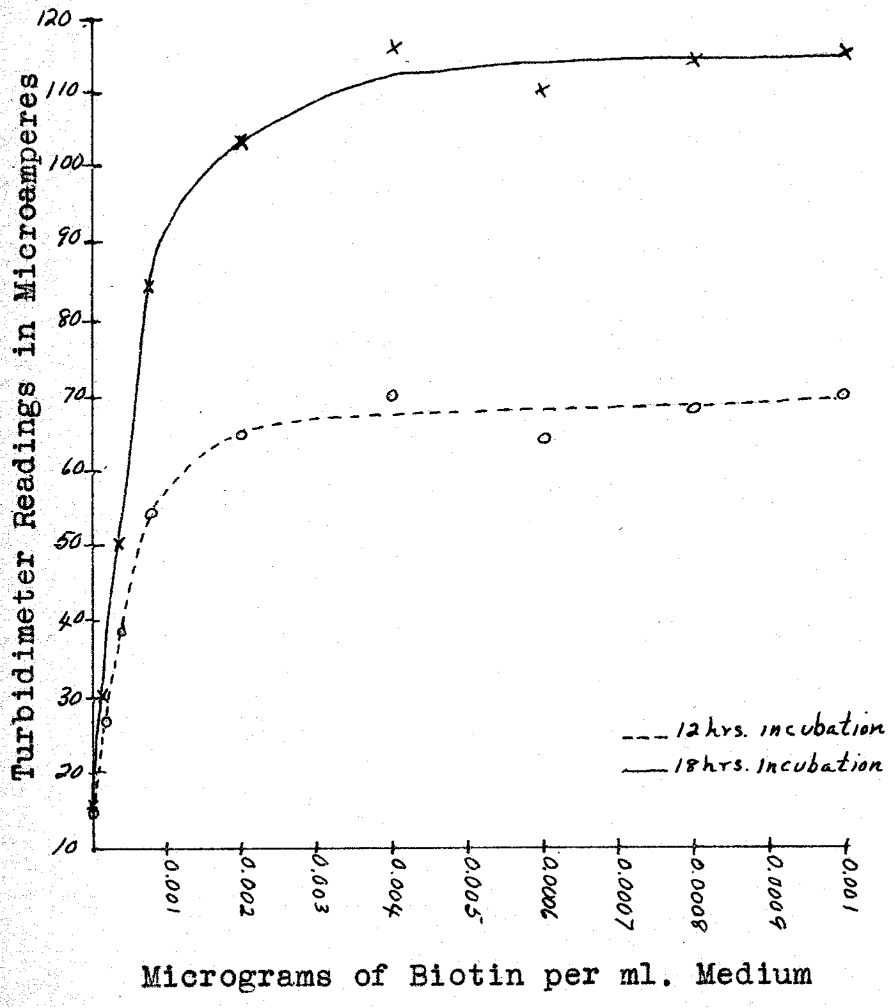


Table 27 (Continued)

Effect of Biotin

Micrograms Biotin per ml. medium	Turbidimeter Readings 12 hrs.	18 hrs.
0.002	69	118
0.003	71	117
0.004	71	113
0.005	66	112
0.006	46	106
0.007	62	111
0.008	48	106
0.009	50	106
0.01	48	100
0.1	48	100

Growth factors constant in all tubes: choline chloride 5.0 µmgs./ml., nicotinic acid 10 µmgs./ml., clacium pantothenate 0.5 µmgs./ml.

Maximum growth was obtained with as little as 0.0004 microgram and a change in growth was produced with 0.00002 microgram biotin per ml. of medium. Slight inhibition of growth was observed in the 12 hour readings with concentrations greater than 0.005 micrograms per ml. medium. This exceedingly small effective concentration is comparable to that determined for brucella (58), rhizobium (59) and hemolytic streptococci (60).

The report of Du Vigneaud et al. (61) on the possible role of pimelic acid was a precursor for biotin in certain diphtheria bacillus stimulated an interest in determining a similar action in the Pneumococcus. There was no measurable turbidity when pimelic acid in concentrations from 0.002 to 0.5 micrograms per ml. medium were substituted for biotin and no additive effect when added to suboptimal concentrations of biotin. Apparently this Pneumococcus is not able to synthesize biotin from pimelic acid.

Nicotinic Acid

As has been mentioned inthe discussion of the biotin concentrates, nicotinic acid was thought to be non-essential for the growth of this organism. That it was a necessary constituent is shown in Table 28 where the effects of varying concentrations of nicotinic acid from 0.01 to 25 microgram per ml. medium are given. See also Graph II

Table 28

Effect of Nicotinic Acid

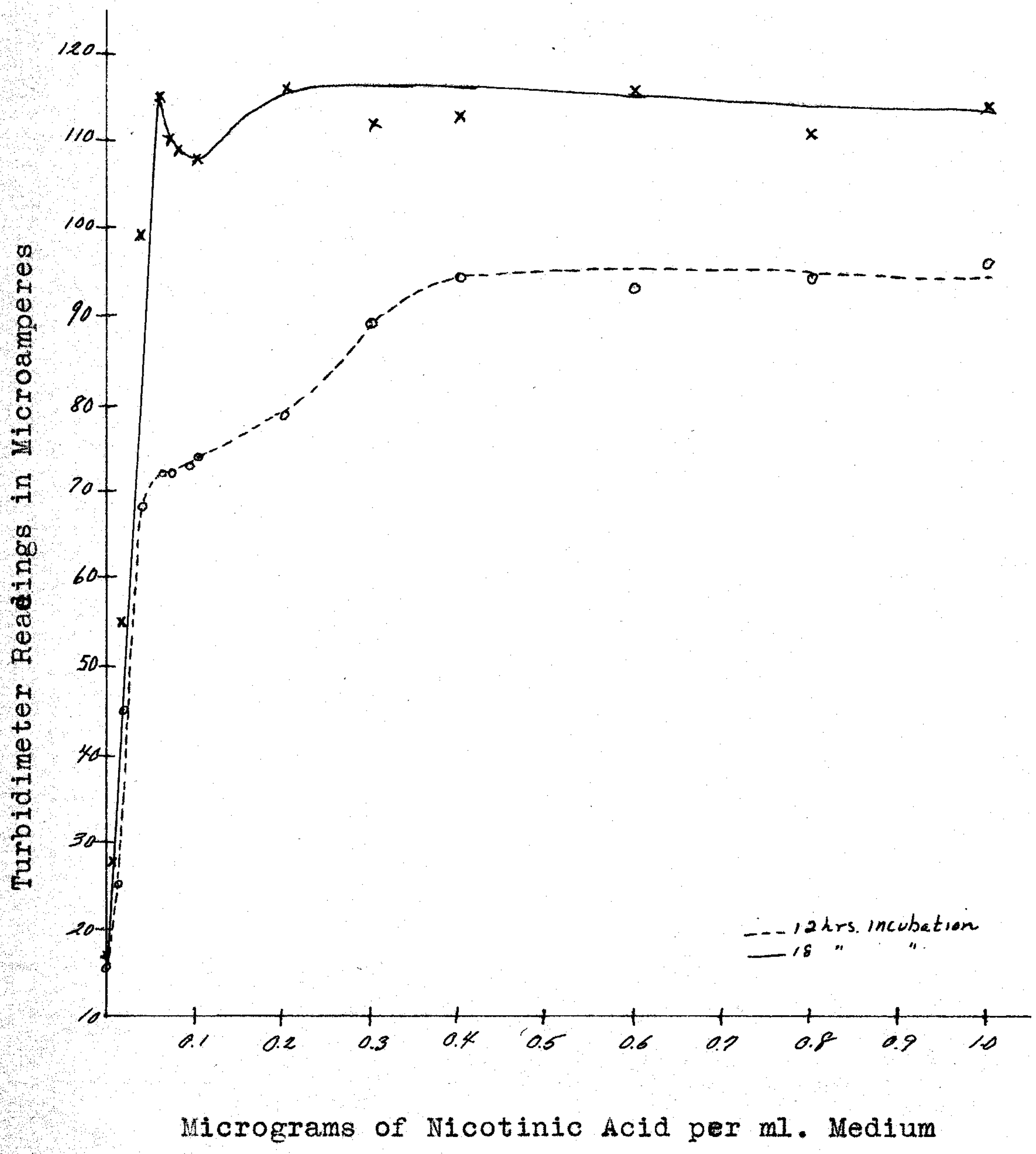
Nicotinic Acid micrograms per ml. medium	Turbidimeter Readings	
	12 hrs.	18 hrs.
0	15	17
0.01	25	28
0.02	45	55
0.04	68	99
0.06	72	115
0.07	72	110
0.08	73	109
0.1	74	108
0.2	79	116
0.3	89	112
0.4	104	113
0.6	103	116
0.8	104	111
1.0	106	114
5.0	106	108
10.0	108	117
15.0	104	115
25.0	98	108

Growth factors constant in all tubes: crystalline biotin 0.001 µmg./ml., calcium pantothenate 0.5 µmg./ml., choline chloride, 5.0 µmgs./ml.

From the table one can see that the most rapid growth was obtained with a minimum concentration of 0.4 microgram nicotinic acid per ml. medium. Maximum turbidity at the

Graph II

Effect of Nicotinic Acid on the Growth of a Type III Pneumococcus



18 hour period was obtained with half that concentration. This effect of suboptimal concentrations of nicotinic acid was also observed when the SMACO biotin concentrates were used with no added nicotinic acid. The same maximum turbidity was reached in 36 hrs. in contrast to 18 hrs. or less when nicotinic acid was added.

From Graph II it is seen that growth is a straight line function of the concentration of nicotinic acid from 0 to 0.04 micrograms per ml. medium. The plateau or dip in the curve from 0.05 to 0.1 micrograms has been obtained every time the nicotinic acid concentration was assayed. The concentration 1.0 microgram of nicotinic acid per ml. medium has been chosen as adequate but not excessive for this organism.

That nicotinamide can replace nicotinic acid is shown in Table 29.

Table 29

Comparison of Growth with Nicotinamide and Nicotinic Acid

Nicotinamide micrograms per ml. medium	Turbidimeter Readings		Nicotinic Acid micrograms per ml. medium	Turbidimeter Readings	
	12 hrs.	18 hrs.		12 hrs.	18 hrs.
0.02	32	44	0.02	45	55
0.04	60	85	0.04	68	99
1.0	90	101	1.0	106	114
5.0	97	102	5.0	106	108

The growth with the nicotinamide appears a little slower when the same concentrations are compared. Even when an excess of 5 mgs. per ml. medium is used there is a significant difference in the turbidity at the end of 12 hrs. incubation.

Pantothenic Acid

The titration of pantothenic acid showed a minimum effective concentration of 0.2 microgram per ml. medium. See Table 30 and Graph III.

Table 30

Effect of Pantothenic Acid

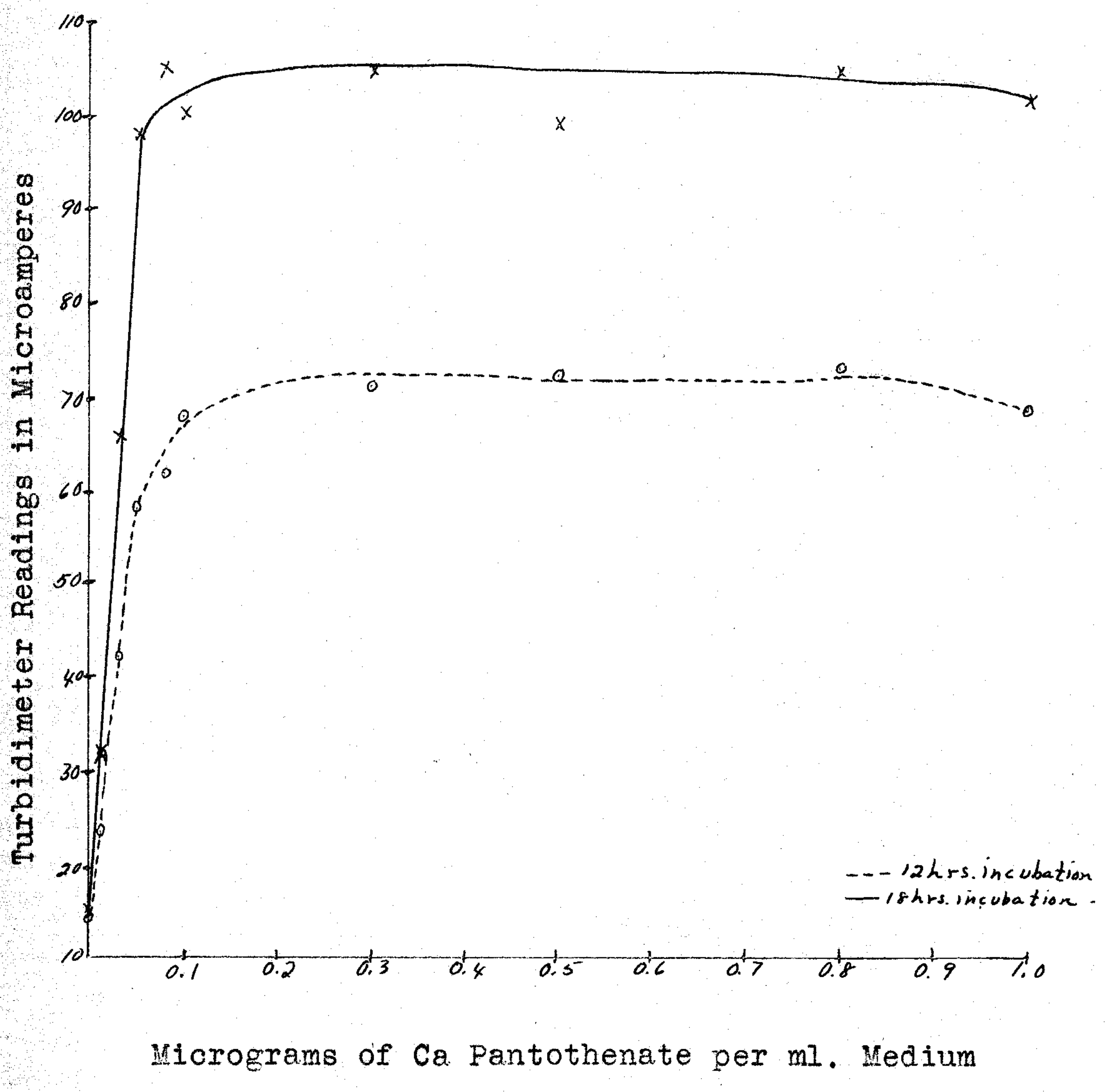
Calcium Pantothenate micrograms/ml. medium	Turbidimeter Readings	
	12 hrs.	18 hrs.
0	14	13
0.005	19	18
0.01	24	32
0.03	42	66
0.05	58	98
0.08	62	105
0.1	68	100
0.3	71	105
0.5	72	99
0.8	75	105
1.0	69	102

Growth factors constant in all tubes: crystalline biotin 0.001 µmg./ml. medium, nicotinic acid 10 µmg./ml., choline chloride 5.0 µmgs./ml.

The slight inhibition suggested at the 12 hr. period with 1.0 microgram has been confirmed in other experiments in which the concentration was carried to 5 microgram per ml. medium.

Graph III

Effect of Pantothenic Acid on the Growth of a
Type III Pneumococcus



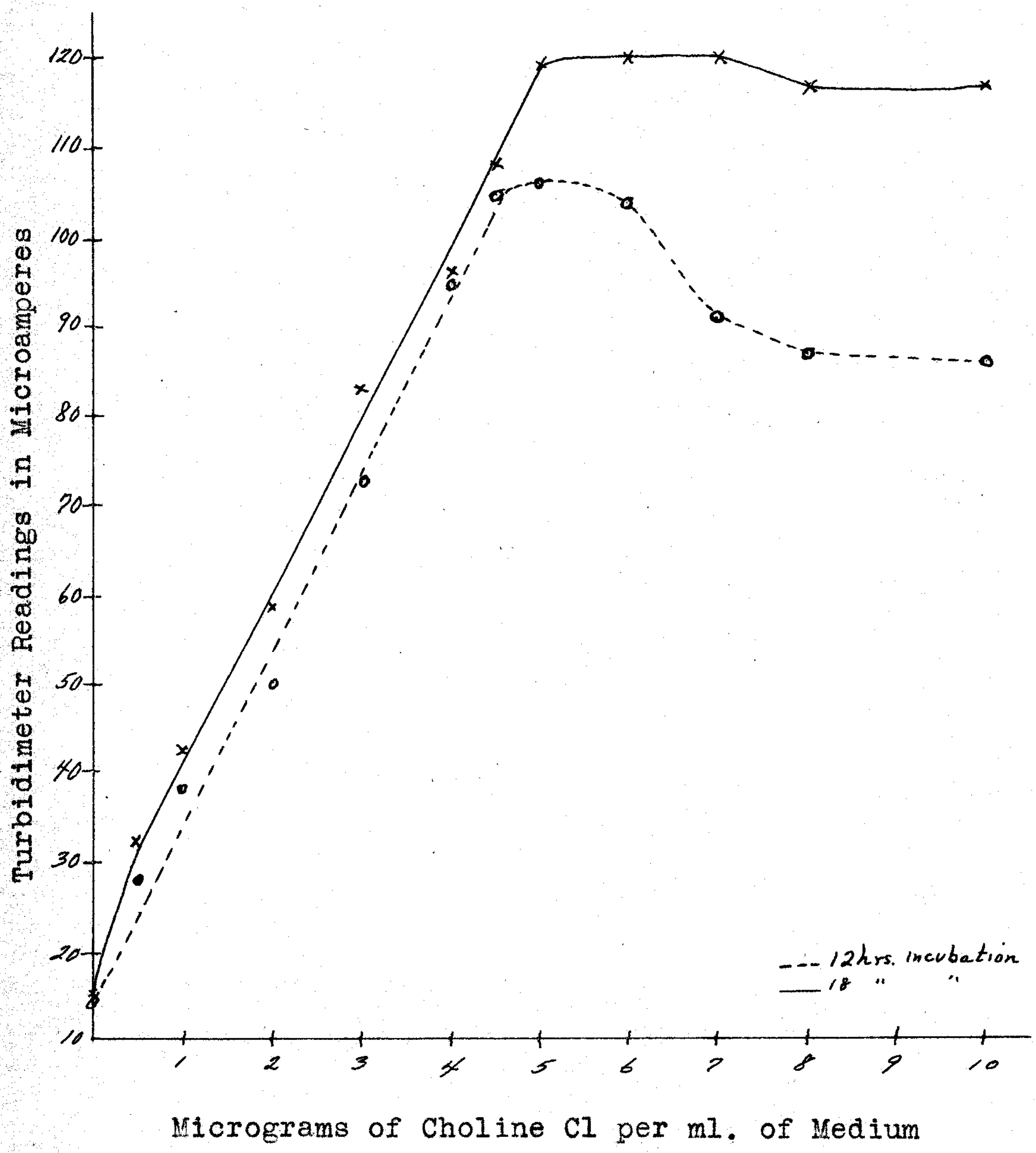
Stiller et al. (62) showed that the pantothenic acid molecule is made up of β-alanine and α-hydroxy-β, β-dimethyl-γ-butyrolactone. β-alanine alone would not replace pantothenic acid in this medium. Apparently this Pneumococcus is unable to synthesize the required pantothenic acid from β-alanine. This is in agreement with the findings of Rane and Subbarow (24) that hydrolysis of the pantothenic acid prevented growth of strains of type I, II, V and VIII Pneumococcus.

Choline

Choline titrations have been run many times with the same optimum concentration of 5.0 micrograms per ml. medium indicated. Typical results are given in Table 31 and Graph IV. The curve in Graph V was obtained with an inoculum prepared from a culture which had been subcultured twenty-four times in the semi-synthetic medium.

Graph IV

Effect of Choline on the Growth of a Type III Pneumococcus



Graph V

Effect of Choline on the Growth of a Type III Pneumococcus after 24 Subcultures

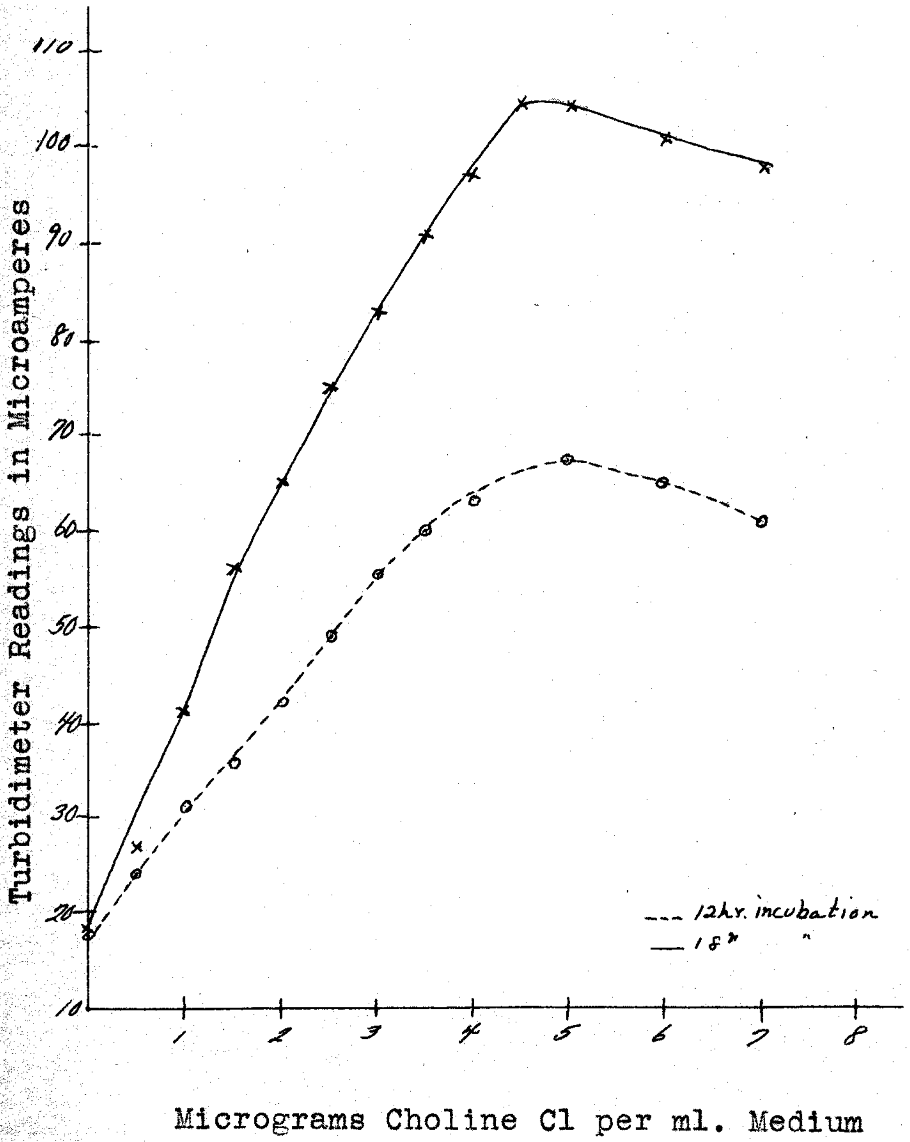


Table 31

Effect of Choline

Choline Chloride micrograms/ml. medium.	Turbidimeter Readings	
	12 hrs.	18 hrs.
0	14	14
0.1	17	19
0.3	22	27
0.5	27	32
0.7	32	34
1.0	38	42
2.0	50	59
3.0	73	63
4.0	95	96
4.5	105	108
5.0	106	119
6.0	104	120
7.0	91	120
8.0	87	117
10.0	86	117
20.0	76	112
30.0	74	107

Growth factors constant in all tubes: Crystalline biotin 0.001 µmg./ml. medium, nicotinic acid 1.0 µmg./ml medium, calcium pantothenate 0.5 µmg./ml.

As has been previously mentioned, the ethanolamine part of the choline molecule will support growth of this organism in the absence of choline. Table 32 shows the growth obtained when ethanolamine was substituted for choline and when it was added to suboptimal concentrations of choline.

Table 32

Comparison of Growth with Choline and Ethanolamine

Choline Chloride micrograms/ml. medium	Ethanolamine micrograms per ml. medium	Turbidimeter Readings		Chain Formation
		11 hrs.	18 hrs.	
3		57	84	-
6		68	122	-
	2.2	20	49	+
	4.4	28	67	+
3	4.4	53	69	-
	11	46	88	+
3	15	58	93	-
	22	51	101	+
	33	51	100	+
6	33	58	103	-
	44	41	92	+
	55	40	89	+

A comparison of the turbidities after 11 hours incubation shows that the maximum turbidity obtained with ethanolamine is not quite equal to that obtained with choline. The most active concentration of ethanolamine is four to six times that of choline and on a molecular basis approximately ten times as much ethanolamine is required to produce an equivalent amount and rate of growth.

When ethanolamine was substituted for choline the resulting growth appeared grossly and microscopically different. With choline the turbidity was diffused and distributed evenly and when observed under the microscope the organisms appeared as discrete diplococci, no chain formation in young or old cultures. While with ethanolamine the turbidity resembled that obtained with choline except when the cultures were swirled, there was a corresponding swirling of the turbidity. This effect was explained when the cultures were examined microscopically and the organisms were found only in long chains of 30 or more diplococci. No single diplococci have been observed. No chain formation has been seen in medium containing both ethanolamine and choline. This difference in type of growth may account for the lower measurable turbidity.

Morgan and Avery (63) have stated that actively growing cultures were indicated by the length of the chain when stained. Dr. L. H. Schmidt* has observed the formation of long chains by this organism when grown in an unfavorable medium where the growth was slow and poor. That the determining factor with ethanolamine was not the rate of growth was shown by the almost equal turbidimeter readings after 11 hrs. incubation.

In connection with this chain formation it is interesting to note the observation of Okamoto (64) of characteristic long chain growth of pneumococci grown on blood agar plates after the addition to the culture media of choline, tetramethylammonium iodide, tetraethylammonium

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* Personal Communication.

iodide, hydrastinine hydrochloride cotarnine hydrochloride, diethylaminoethanol hydrochloride, triethanolamine hydrochloride or triethylamine hydrochloride.

A more detailed chemical and immunological study of this phenomenon would undoubtedly yield some interesting information.

Summary of Quantitative Studies.

In determining the minimum or optimum effective concentrations of growth factors, it is necessary to keep in mind the possible effect of the ratio of the amounts of one factor to another and to various ingredients of the medium. The quantitative results obtained with this medium might vary considerably if the concentrations of the various basic ingredients were changed appreciably. This fact must be kept in mind in working out the specific amino acid requirements. Until that time the medium is not synthetic and further quantitative studies previous to the development of this synthetic medium seemed superfluous.

The results of the quantitative studies may be summarized in Medium VI. A consistent rapid luxurient growth was obtained with this medium.

Medium VI

Casein acid hydrolysate	0.5	mgs.N per ml. medium
l cystine	25.0	micrograms
KH_2PO_4	5.0	mgs.
$MgSO_4.7H_2O$	0.5	mgs.
Choline chloride	5.0	micrograms
Pantothenic acid	1.0	"
Nicotinic acid	1.0	"
Biotin (crystalline)	0.001	"
Thiamine	0.5	"
Creatine	5.0	"
Uracil	5.0	"
Glutamine	150	"
Ascorbic acid	300	"
Glucose	5	mgs.

Bacteriological Studies

To test the completeness of this medium, the study of various bacteriological aspects of growth in Medium V was undertaken.

Inoculum

Since the inoculum used throughout this work had been so large it was necessary to demonstrate that no growth factors were introduced through it. This had partially been indicated by the slight growth resulting from the omission of any one of the essential growth factors, but further proof was necessary. This proof was offered by two different experiments: first, by successive subcultures and second, by inoculating with only a few organisms.

Growth in successive subcultures in Medium V was demonstrated in the following way. The original inoculum was prepared as described in the methods and 0.1 ml. used to inoculate the first subculture in the semi-synthetic medium. After 12 hours incubation at 34°C. the first subculture was placed in the refrigerator for 12 hours, then 0.1 ml. was inoculated into the second subculture. This procedure was repeated through 40 subcultures after which it was no longer convenient to continue it. After passing through forty subcultures the rate and extent of growth remained the same and the organism still retained its type specificity as determined by a positive Neufeld reaction.

To test the survival and multiplication of only a few organisms in this medium, the inoculum, prepared in the usual manner, was diluted serially in sterile basal medium and 0.1 ml. of each dilution was inoculated into three tubes containing 10 ml. of Medium V. The higher dilutions were plated out and the number of organisms in the inocula estimated from the plate counts. Turbidity measurements were made every hour up to 14 hours, every two hours up to 24 hours and then after 27, 45 and 50 hours incubation. The hour at which maximum turbidity was observed is given. A summary of the results is given in Table 33.

Table 33

Effect of the Size of Inoculum

Number of Organisms inoculuated into 10 ml. medium	Hours of Incubation to produce maximum turbidity	Maximum Turbidits readings
20,000,000	18	100
2,000,000	24	99
200,000	27	102
20,000	45	102
2,000	45	104
200	45	104
20	45	101
2	50	102
2*	50	101

* Growth in two of the three tubes

Stained smears from each tube showed pure cultures of Pneumococci. From this it was concluded that all the substances required for the growth of this organism were present in the medium and were not introduced by the inoculum. Further evidence of the completeness of the medium was given by growth of the organisms on agar plates prepared by adding 1.2 per cent of Bacto agar to Medium V. Colonies appeared within 18 hours and were opaque and elliptical in shape.

Viability

From a practical viewpoint it was interesting to determine the viability of the organisms for any length of time when grown and stored in this medium. Tubes containing one-half the usual glucose concentration were incubated 12 hours at 37°C. and stored in the refrigerator. Subcultures from these stored cultures were made at weekly intervals. Viable, type specific organisms were still present at the end of four weeks. By a more careful control of the pH, age and exposure to atmospheric oxygen of the culture, the period of survival could probably be extended.

Virulence

A complete medium for a pathogenic organism must also maintain the virulence. That the cultural medium has much to do with the maintenance of pneumococcal virulence has been demonstrated by Felton _et al._ (65). Consequently it was of interest to determine the virulence after several successive transfers in this medium.

Serial dilutions of a 12 hour culture which had been subcultured twenty-three times in Medium V, were made in sterile basal medium. Four mice were injected intraperitoneally with 1 ml. of each dilution. The number of organisms injected were determined by plate counts. Smears made from the heart blood of all animals that died showed Pneumococcus when stained. The results are presented in Table 34.

Table 34

Virulence of a Type III Pneumococcus after Twenty-Three Subcultures in a Semi-Synthetic Medium

Dilution of Culture	Number of Organisms Injected	Approximate Time of Death in Hrs.
10^{-4}	100,000	24, 28, <42, <42
10^{-5}	10,000	25, 27, 29, <42
10^{-6}	1,000	29, <42, <42, <42
10^{-7}	100	42, <50, <50, <50
10^{-8}	10	42, <50*

* Had not died at the end of two weeks.

Unfortunately the dilution which contained one organism per ml. was not injected into mice but two out of four mice receiving ten organisms died. From this preliminary experiment it may be concluded that the virulence was maintained after twenty-three subcultures in the semi-synthetic medium.

According to White (25, p. 199) the average minimal lethal dilution for all Type III strains is 2 to 100,000,000.

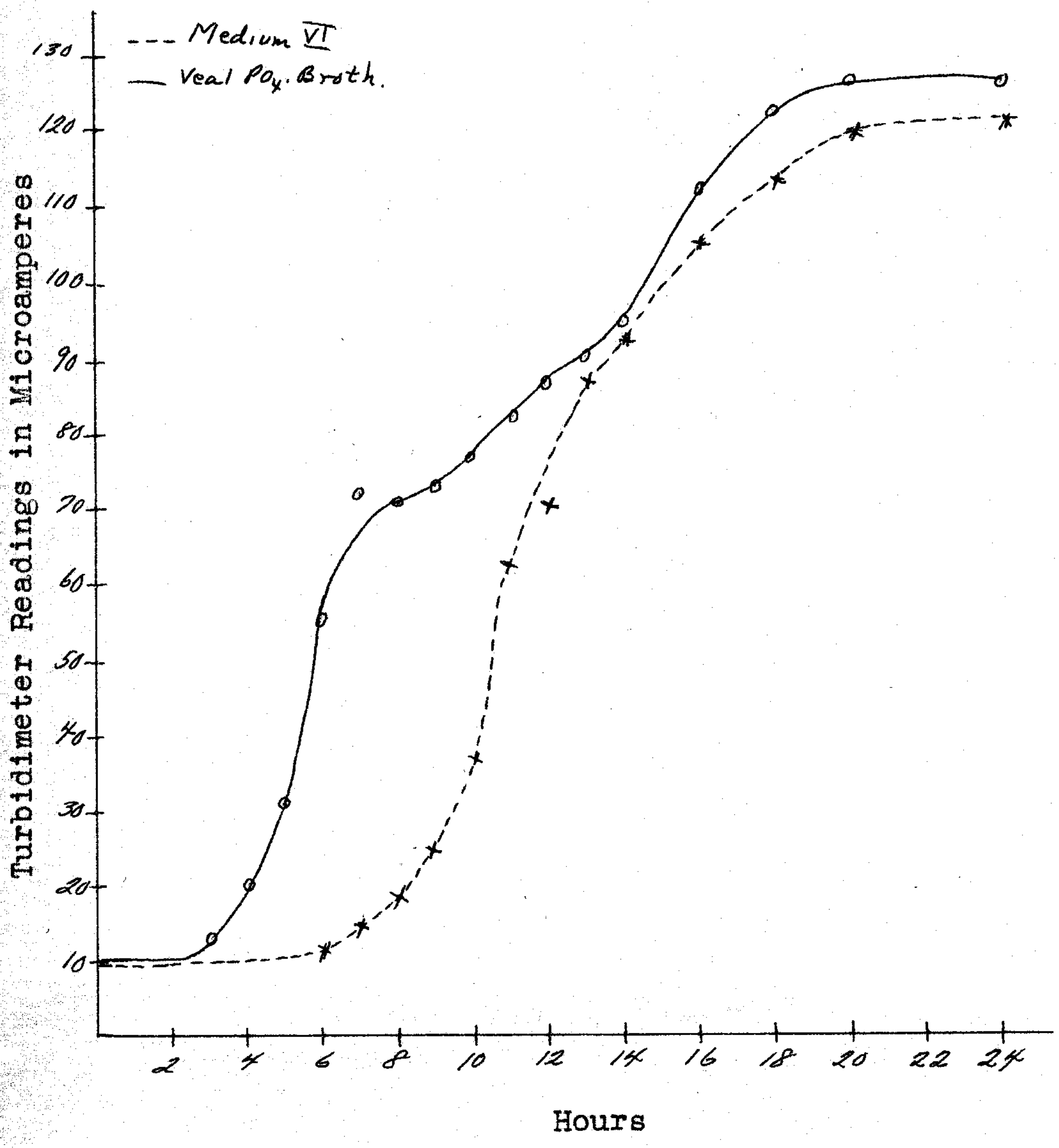
Comparison of Growth in Medium V to that in Veal Phosphate Broth.

Growth in the semi-synthetic medium compared favorably with growth in veal phosphate infusion broth in all respects except one. The lag period in the simpler medium was longer than in the complex veal broth. This was brought out clearly when growth curves of the two different media were compared. (Graphs VI and VII). Two different inocula were used, one of 2,000,000 organisms per ml. and the other with 200,000 organisms per ml. of medium. Triplicate tubes were run for each inoculum and turbidimeter readings were taken every hour for 14 hours, then every two hours for ten hours. The incubation was carried out at 34°C. and the glucose content of both media was 5 mg. per ml. of medium. The number of organisms in the inocula was calculated from plate counts. At the end of two and three hours incubation, serial dilutions with plate counts were made from a tube of semi-synthetic medium. The culture before incubation contained 1-2,000,000 organisms per ml. At the end of two hours incubation the population was 3-4,000,000 and at the end of three hours incubation the population was 6-7,000,000, indicating that during this period previous to the appearance of turbidity the organisms

Graph VI

Comparison of Turbidity in Medium VI and Veal Phosphate Broth

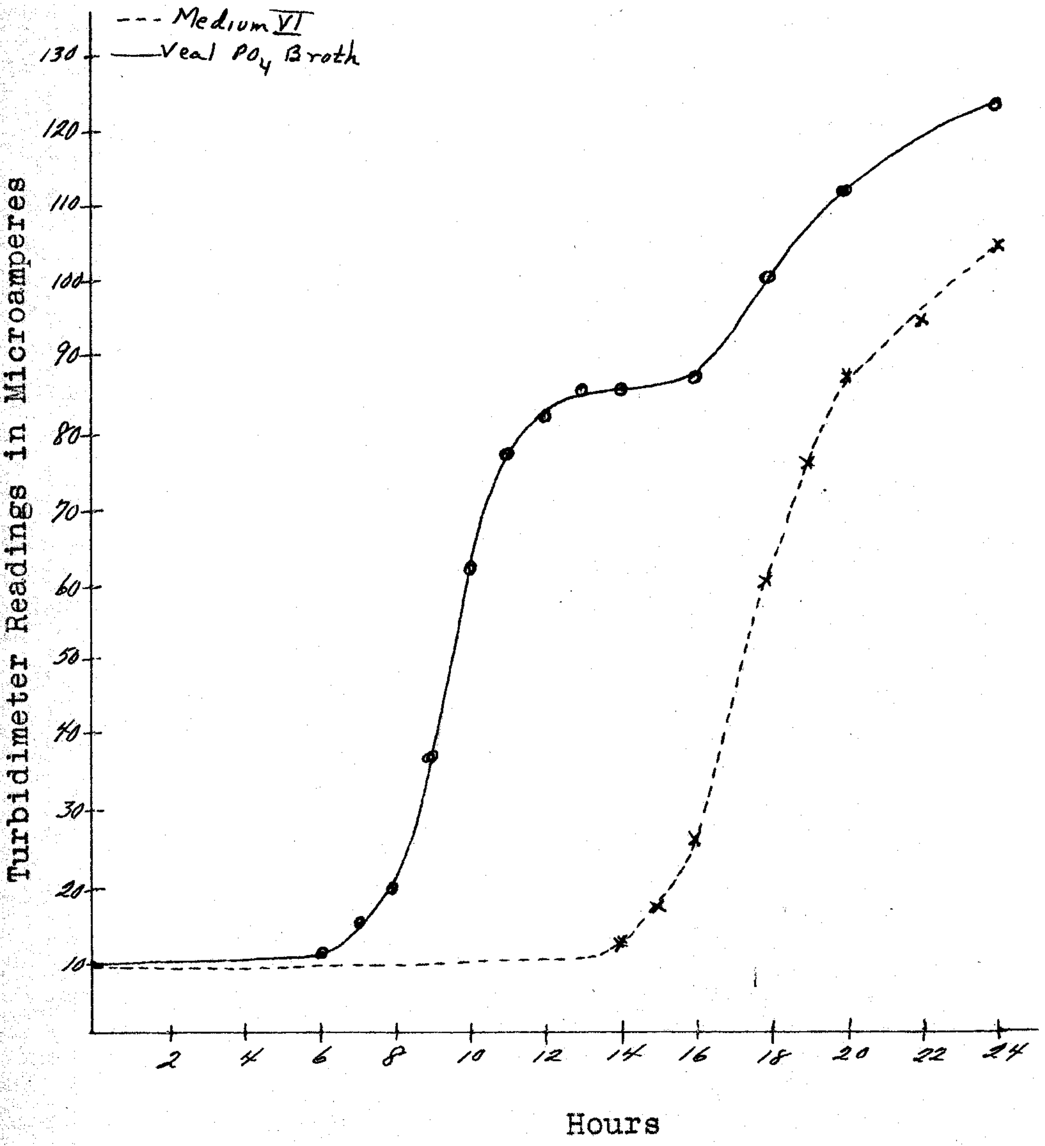
Inoculum = 2,000,000 Pn. per ml.



Graph VII

Comparison of Turbidity in Medium VI and Veal Phosphate Broth

Inoculum = 20,000 pn. per ml.



were not dying.

This lag period may have been due to a number of factors: unfavorable oxidation-reduction potential, unfavorable concentration of organic or inorganic constituents of the medium, absence of some growth accelerating substance, or the presence of some toxic substance. Investigation of these factors is planned.

Metabolic Studies

For assay purposes, the possibility of using the measurement of acid production as a measure of growth was investigated. Titration of acidity has the desirable advantage of requiring no special equipment.

An experiment was planned to compare acidity and turbidity of cultures containing varying amounts of choline. At the end of 12 hours the turbidity was measured by the Turbidity Comparator and the acid produced was titrated directly in the tubes with 0.1 N NaOH, using phenolphthalein as indicator. A second set of tubes was incubated 36 hours and the turbidity and acidity then measured. The values shown in Table 35 and Graph VIII are averages of duplicate determinations.

Graph VIII

Comparison of Acidity and Turbidity after 36 Hours Incubation

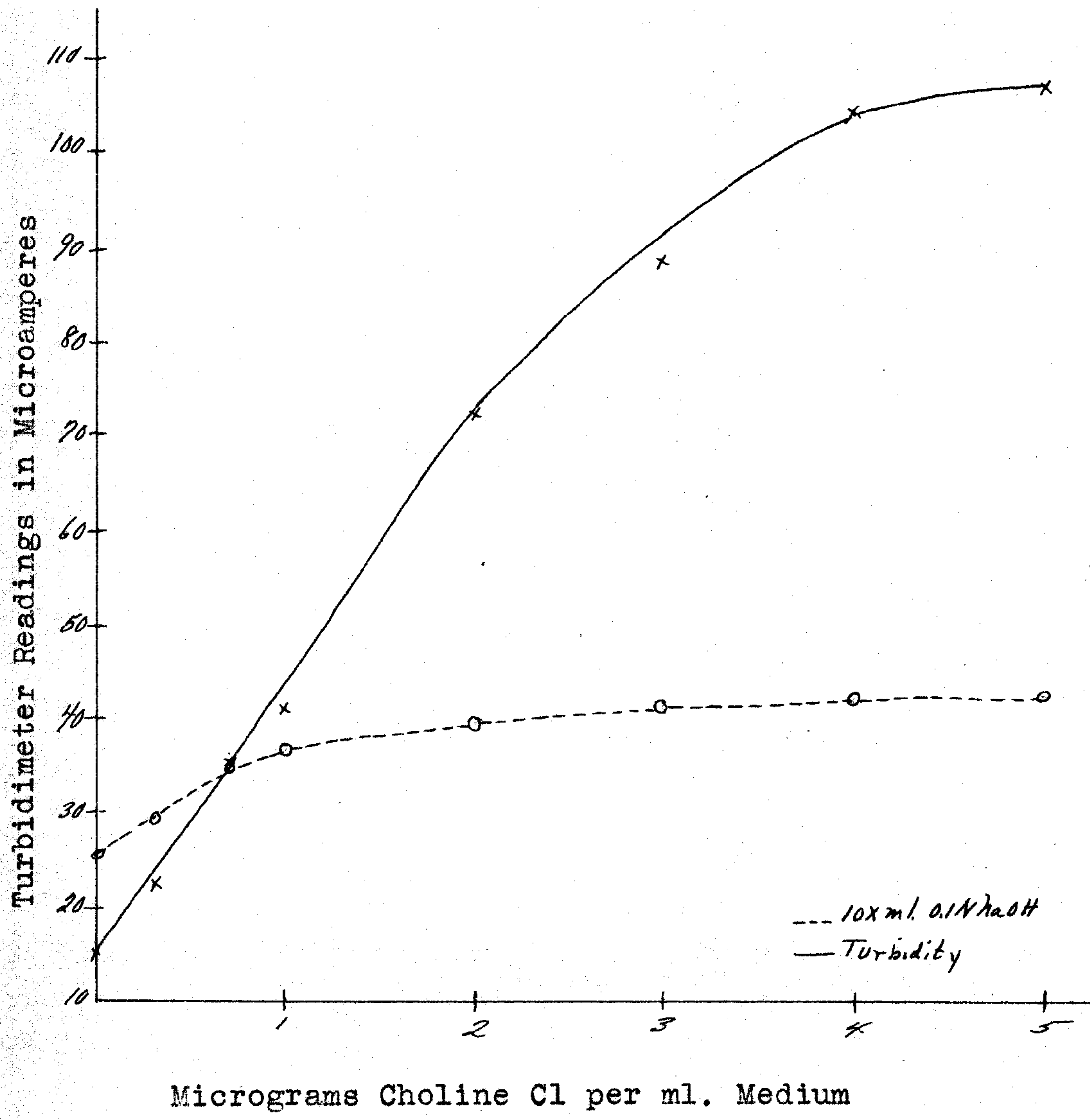


Table 35

Comparison of Turbidity and Acid Production of Cultures Containing Various Concentrations of Choline

	Micrograms Choline per ml. Medium	Turbidimeter Readings	mls. 0.1 N NaOH
<u>12 hrs.</u>			
	0	12	1.26
	0.1	13	1.40
	0.5	19	2.04
	0.9	26	2.23
	1.5	32	2.59
	2.5	42	3.18
	3.5	45	3.44
	4.5	46	3.60
<u>36 hrs.</u>			
	0	15	2.50
	0.3	22	2.96
	0.7	35	3.48
	1.0	41	3.62
	2.0	72	3.98
	3.0	89	4.19
	4.0	104	4.23
	5.0	107	4.23

After studying the rather unexpected results it was seen that the titratable acidity was too small to use for assay purposes. The most outstanding fact was the difference of the slopes of the turbidity and acid production curves. Several explanations were considered: (1) the cessation of acid production due to lack of carbohydrate, (2) the metabolism of the acid end products by adaptive enzymes, (3) the increase in turbidity at the end of 36 hours not due to increase in the number of organisms.

Experiments were designed to study these different possibilities. Additional glucose was added to culture tubes but the total acidity remained the same.

mgs. Glucose per ml. Medium	mls. 0.1 N NaOH
2.5	2.65
5	4.20
7.5	4.20
10.0	4.20

It had already been shown that maximum turbidity was obtained with as little as 3 mgs. glucose per ml. of medium; but the acid formed had not been measured.

Friedemann (66) has found that the principal products under aerobic and anaerobic conditions of Pneumococcus grown 24 hours at 37°C. in meat infusion medium enriched with 1 per cent peptone, 0.7-1.8 per cent $NaH_2PO_4.12\ H_2O$ and 1 per cent glucose were lactic acid, formic acid, acetic acid and ethyl alcohol. The lactic acid accounted for about 80 per cent of the glucose metabolized. When lactic acid and pyruvic acid were substituted for glucose or were added with suboptimal concentrations of glucose no effect on turbidity or acid production was observed. Neither pyruvic acid or lactic acid served as partial or complete carbohydrate source for this organism under the described experimental conditions. The addition of lactic acid, formic acid, acetic acid and ethyl alcohol to the uninoculated medium produced no apparent change.

Favorable evidence for the third theory was given by reports in the literature of the relation of Pneumococcus to hydrogen ion concentration in complex cultural media (29). It is generally agreed that "the most important bactericidal factor in the growth and death of the Pneumococcus in fluid media containing 1 per cent glucose is the production of acid. At a pH of 5.1 or lower the Pneumococcus does not survive longer than a few hours." Does this also hold for Pneumococci grown in a semi-synthetic medium.

An experiment was planned to compare turbidity, acid production and numbers of viable organisms in a growing culture. Forty tubes of medium V were set up, inoculated in the usual manner and incubated at 37°C. At hourly intervals two tubes were removed, the turbidity measured and 0.1 N NaOH added until the culture was neutral to phenolphthalein. Plate counts were made after 0, 6, 12 and 18 hours incubation. The results are given in Table 36 and Graph IX.

Graph IX

Comparison of Acidity and Turbidity During the First Twenty-four Hours Incubation

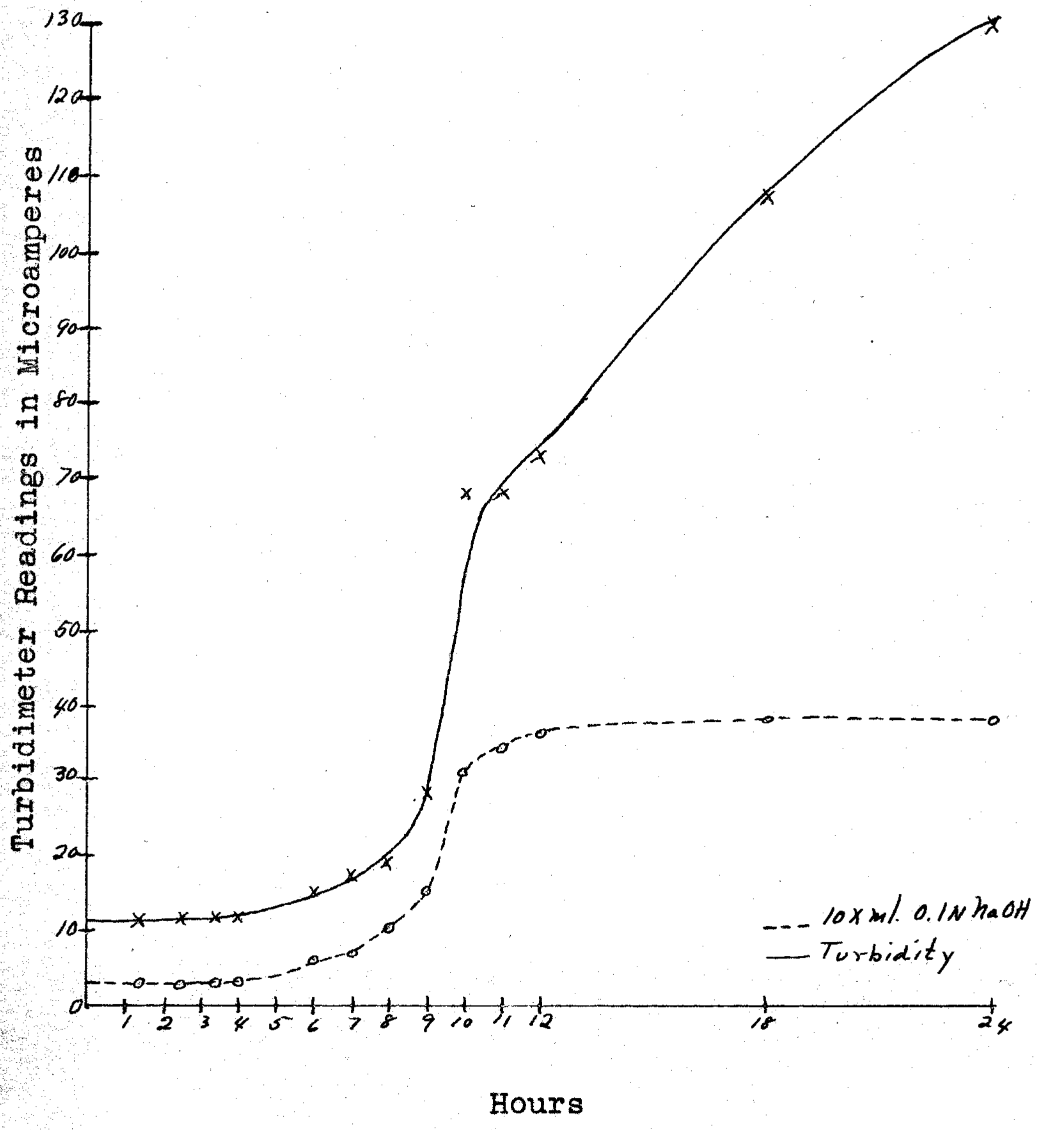


Table 36

Comparison of Turbidity, Acid Production and Number of Viable Organisms Grown on Medium V

Hours Incubated	Turbidimeter Readings	mls. 0.1 N NaOH	Organisms per ml. of Medium
0	12	0.35	7,000,000
1.5	12	0.35	
2.5	13	0.35	
3.5	12	0.36	
4.5	12	0.36	
5.75	15	0.64	
6	16	0.65	200,000,000
7	16	0.73	
8	19	1.09	
9	28	1.52	
10	68	3.15	
11	68	3.46	
12	173	3.62 (pH = 5.1)	1,000,000,000
18	107	3.80 (pH = 5.0)	100,000,000*
24	130	3.84 (pH = 5.0)	
30	133		
44	131		

* No growth in any of the plates made at 18 hours. Lowest dilution plated was 10^{-8}.

From Graph IX it appears that up to about 12 hours of incubation the turbidity and acid production curves were almost parallel, but after this time the turbidity continued to increase at a different rate and the acid production stopped. The number of viable organisms increased up to 12 hours, then decreased. To obtain a clearer picture of the period between 12-18 hours incubation a more detailed study was planned. In this, the turbidity, number of viable organisms and total number of organisms were compared (Table 37). Change in the total number of organisms was based on total nitrogen determinations of the centifuged and washed sediment from the cultures. Tubes of medium were inoculated at the same time and with the same inoculum as the plates, thus testing any possibility of an apparent decrease in viable organisms due to inability to grow on solid medium. There was excellent correlation. Dilutions at which colonies appeared on the plates showed corresponding growth in the liquid medium. Dilutions at which no colonies were seen on the plates showed no growth in the liquid medium.

Table 37

Comparison of Turbidity, Number of Viable Organisms and Total Nitrogen of 12 to 18 hours Cultures Grown in Medium VI

Hours Incubated	Turbidimeter Readings	Number of Viable Organisms per 10 ml.	Mgs. Nitrogen per 10 ml.
12	66	10,000,000,000	0.510 0.516
14	89	6,000,000,000	0.503 0.498
16	98	400,000,000	0.489 0.493
18	110	500,000	0.506 0.503

From these experimental data the growth and death of Pneumococci in a semi-synthetic medium containing glucose appeared to be essentially the same as in the more complex media. The organisms multiplied and produced acid until the acidity of the medium became so great that the organisms began to die. That these dead organisms were not dissolved to any great extent was indicated by the constant total nitrogen of the sediment.

To what then was the increased turbidity due during this period? If from the constant nitrogen it may be concluded that there was no increase in number of organisms, then the only remaining change which could be correlated with this increased turbidity was the death of the organisms. Perhaps the organisms at this pH disintegrated producing more particles, thus more deflection of light and increased turbidity. In this event the nitrogen of the particulate matter and the acidity would remain constant as the organisms died and the turbidity increased.

Experiments of Lord and Nye (34) on the dissolution of the Pneumococcus substantiate this theory. Cloudy suspensions of washed Pneumococci in solutions of varying pH from 8.0 to 4.0 showed after incubation, dissolution of organisms at pH values higher than 5.0. No dissolution occurred at the most acid end of the scale. Change in the organism was also evidence by the fact that Pneumococci became bile-insoluble after remaining at pH for two hours.

The question immediately arose as to the interpretation of the turbidimeter values. According to Cullen and Chesney (68), the change in pH of the medium paralleled change in the rate of growth of Pneumococcus, and according to Lord and Nye (34) the greater the acidity, the more rapid the death. If the turbidity up to a certain time was due to growth of organisms and after that time the increase in turbidity was due to the death of the organisms, then the turbidimeter values taken during this dynamic period should give a comparison of the extent and rate of growth. Thus a turbidimeter value after 18 hours of 100 compared to 120 would indicate that in the tube reading 120, growth was initiated earlier or that it proceeded at a faster rate.

In the titrations of the necessary growth factors, turbidity was definitely a function of the growth of the organisms. This was shown in an experiment in which the total nitrogen was determined after 18 hours incubation on cultures with varying concentrations of choline. The results are presented in Table 38.

Table 38

Comparison of Turbidity and Total Nitrogen of Cultures Grown in Various Concentrations of Choline.

Choline Chloride micrograms per ml. medium	Turbidimeter Readings 18 hours	Mgs. Nitrogen per 10 mls. culture
2	44	0.386 0.388
3	67	0.401 0.403
4	83	0.452 0.455
5	99	0.501 0.496

Before the growth of this organism could be used for assay purposes, the turbidity would have to be standardized by the determination of bacterial nitrogen.

Study of the Choline Requirements.

In the study of the structural specificity of choline and ethanolamine for the growth of this Pneumococcus, the compounds tested have been arranged according to the atom of the ethylamine moiety on which substitution occurred.

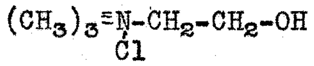
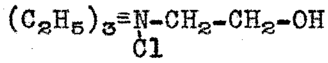
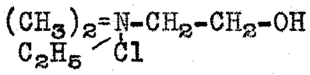
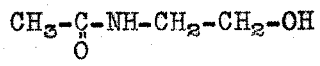
Table 39 Derivatives of the nitrogen atom
Table 40 Derivatives of the first carbon atom
Table 41 Derivatives of the second carbon atom

The methods used in determining the growth activity of the different compounds were the same throughout. The material to be tested was added to the medium without choline in molecular concentrations equal to the effective concentrations of choline. If growth resulted, the experiment was repeated until the concentration at which maximum growth occurred was determined. If no growth resulted from the addition of the compound, a wider range of concentrations was tried. The compounds were also added to tubes containing suboptimal and optimal concentrations of choline to test the additive as well as any stimulating or inhibiting effects. None of the compounds inhibited or accelerated growth in the presence of optimal concentrations of choline. Control tubes with and without choline were also run with each experiment. The turbidity was used as a comparison of growth and readings were made at the end of 12, 18 and 24 hrs. incubation. Where there was slight or no growth, the cultures were allowed to incubate at least 36 hrs. to allow time for slow utilization to become apparent. In general, the picture of activity shown within 12 hrs. was unaltered qualitatively or quantitatively on further incubation. The concentrations producing maximum turbidity are expressed in equivalents of choline in Tables 39, 40 and 41. The activities of the different compounds are expressed in percentages of the turbidity obtained with 5 micrograms of choline after 12 hrs. incubation. The compounds are listed according to their activities in Tables 42 - 45.

An analysis of the compounds obtained by substitution on the nitrogen atom of the ethylamine moiety, indicated that the trimethyl groups of the choline molecule were not the essential part. Replacement of any one or all of the trimethyl groups with ethyl groups did not alter the ability of the molecule to produce growth. Whether the nitrogen was primary, secondary, tertiary or quarternary seemed to have no relation to the growth activity. The greater activity of the di- and triethanolamine over the monoethanolamine and tetraethanolammonium hydroxide is difficult to explain. Two different sources of the ethanolamine were tested as well as some repurified by crystallization of the oxalate. The results confirmed those given and thus eliminate the factor of possible impurity. The slight activity of N-acetylethanolamine was probably due to ethanolamine formed by hydrolysis of the acetyl group in the slightly alkaline medium. When autoclaved with the medium activity approached that of ethanolamine. The

Table 39

Activity of Ethanolamine and Choline Derivatives
Substitution on the Nitrogen Atom

Name of Compound	Structural Formula	Equivalent conc. μmg./ml.medium	Activity
1) Choline chloride	 $(CH_3)_3\equiv\underset{Cl}{N}-CH_2-CH_2-OH$	5	100
2) Dimethylethanolamine	$(CH_3)_2=N-CH_2-CH_2-OH$	5	100
3) Methyldiethanolamine	$CH_3-N=(CH_2-CH_2-OH)_2$	5	100
4) Triethylcholine chloride	 $(C_2H_5)_3\equiv\underset{Cl}{N}-CH_2-CH_2-OH$	5	100
5) Diethylaminoethanol	$(C_2H_5)_2=N-CH_2-CH_2-OH$	5	100
6) Dimethylethylcholine chloride	 $\underset{C_2H_5}{(CH_3)_2}=\underset{Cl}{N}-CH_2-CH_2-OH$	5	100
7) Ethanolamine	$NH_2-CH_2-CH_2-OH$	50	80
8) Diethanolamine	$NH=(CH_2-CH_2-OH)_2$	10	95
9) Triethanolamine	$N\equiv(CH_2-CH_2-OH)_3$	10	95
10) Tetraethanolammonium-hydroxide	$HO-N\equiv(CH_2-CH_2-OH)_4$	30	85
11) N-acetylethanolamine [1]	 $CH_3-\underset{O}{C}-NH-CH_2-CH_2-OH$	50	23
12) Phenylethanolamine	$NH-CH_2-CH_2-OH$	1-50	0

[1] Added after autoclaving.

Table 40

Activity of Ethanolamine and Choline Derivatives
Substitution on the First Carbon Atom

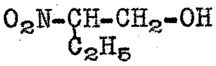
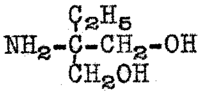
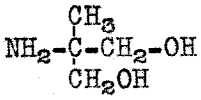
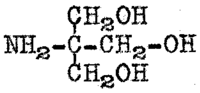
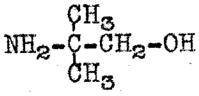
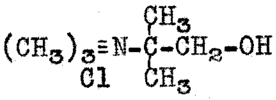
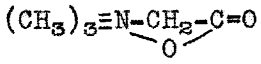
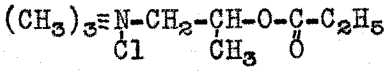
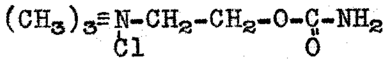
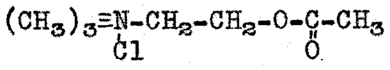
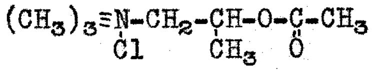
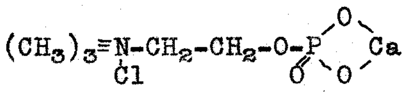
	Name of Compound	Structural Formula	Equivalent conc. μmg./ml.medium	Activity
	Choline chloride		5	100
13)	2-amino-1-butanol	 $NH_2-\underset{C_2H_5}{CH}-CH_2-OH$	5	80
14)	2-nitro-1-butanol	 $O_2N-\underset{C_2H_5}{CH}-CH_2-OH$	1-50	0
15)	2-amino-2-ethyl-1,3-propanediol	 $NH_2-\overset{C_2H_5}{\underset{CH_2OH}{C}}-CH_2-OH$	10	100
16)	3-amino-2-methyl-1,3-propanediol	 $NH_2-\overset{CH_3}{\underset{CH_2OH}{C}}-CH_2-OH$	40	90
17)	2-amino-2-hydroxymethyl-1,3-propanediol	 $NH_2-\overset{CH_2OH}{\underset{CH_2OH}{C}}-CH_2-OH$	50	5
18)	2-amino-2-methyl-1-propanol	 $NH_2-\overset{CH_3}{\underset{CH_3}{C}}-CH_2-OH$	50	31
19)	α,α-dimethylcholine chloride	 $(CH_3)_3\equiv\underset{Cl}{N}-\overset{CH_3}{\underset{CH_3}{C}}-CH_2-OH$	5	20
20)	Serine	 $NH_2-\underset{COOH}{CH}-CH_2-OH$	1-50	0

Table 41

Activity of Ethanolamine and Choline Derivatives Substitution on the Second Carbon Atom

Name of Compound	Structural Formula	Equivalent conc. μmg./ml.medium	Activity
Choline chloride		5	100
21) Ethylamine	$NH_2-CH_2-CH_3$	1-50	0
22) Glycine	NH_2-CH_2-COOH	1-50	0
23) Sarcosine	$CH_3-NH-CH_2-COOH$	1-50	0
24) Ethylenediamine	$NH_2-CH_2-CH_2-NH_2$	1-50	0
25) Betaine	 $(CH_3)_3\equiv \underbrace{N-CH_2-C}_{O}=O$	1-50	0
26) β-Methoxyethylamine	$NH_2-CH_2-CH_2-O-CH_3$	1-50	0
27) Urethane of β-methyl choline chloride	 $(CH_3)_3\equiv\underset{Cl}{N}-CH_2-\underset{CH_3}{CH}-O-\underset{\overset{\|}{O}}{C}-C_2H_5$	1-50	0
28) Carbamylcholine chloride	 $(CH_3)_3\equiv\underset{Cl}{N}-CH_2-CH_2-O-\underset{\overset{\|}{O}}{C}-NH_2$	1-50	0
29) Acetylcholine chloride [1]	 $(CH_3)_3\equiv\underset{Cl}{N}-CH_2-CH_2-O-\underset{\overset{\|}{O}}{C}-CH_3$	5	7
30) Acetyl-β-methyl choline chloride [1]	 $(CH_3)_3\equiv\underset{Cl}{N}-CH_2-\underset{CH_3}{CH}-O-\underset{\overset{\|}{O}}{C}-CH_3$	5	7
31) Phosphorylcholine chloride (Ca)	 $(CH_3)_3\equiv\underset{Cl}{N}-CH_2-CH_2-O-\underset{\overset{\|}{O}}{P}\genfrac{}{}{0pt}{}{\diagup O\diagdown}{\diagdown O\diagup}Ca$	1-50	0

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[1] Added after autoclaving.

Table 41(continued)

Activity of Ethanolamine and Choline Derivatives
Substitution on the Second Carbon Atom

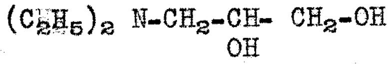
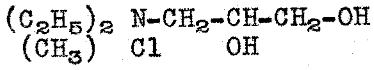
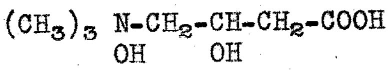
Name of Compound	Structural Formula	Equivalent conc. μmg./ml.medium	Activity
32) γ-Diethylamino-propanol	$(C_2H_5)_2\ N-CH_2-CH_2-CH_2-OH$	6	100
33) 2,3-Propanediol-1-diethylamine	 $(C_2H_5)_2\ N-CH_2-\underset{OH}{CH}-\ CH_2-OH$	10	100
34) Diethylmethyl-β,γ-dihydroxypropyl-ammonium chloride	 $\underset{(CH_3)}{(C_2H_5)_2}\ \underset{Cl}{N}-CH_2-\underset{OH}{CH}-CH_2-OH$	5	20
35) Carnitine	 $(CH_3)_3\ \underset{OH}{N}-CH_2-\underset{OH}{CH}-CH_2-COOH$	1-50	0

Table 42

Compounds Which Give the Same Growth as Choline on an Equimolar Basis

Choline chloride

Dimethylethanolamine

Methyldiethanolamine

Dimethylethylcholine chloride

Triethyl choline chloride

Diethylaminoethanol

Diethanolamine

Triethanolamine

2-Amino-1-butanol

2-Amino-2-ethyl-1,3-propanediol

Table 43

Compounds Which Will Produce Approximately as Much Growth as Choline if Added in Higher Concentrations

Ethanolamine

Tetraethanolammonium hydroxide

2-Amino-2-methyl-1,3-propanediol

Table 44

Compounds Which Produce About One Fifth as Much Growth as Choline Even at Concentrations Above Which There is no Effect Except Inhibition

N-acetylethanolamine

2-Amino-2-hydroxymethyl-1,3-propanediol

2-Amino-2-methyl-1-propanol

Diethylmethyl-β,γ-dihydroxyproplyammonium chloride

α,α-Dimethylcholine chloride

Acetylcholine chloride

Acetyl-β- methylcholine chloride

Table 45

Compounds Which Will Produce no Growth When Substituted for Choline but Which do not Inhibit Growth When Added to the Medium with Choline

Phenylethanolamine

2-Nitro-1-butanol

Serine

Glycine

Ethylamine

Ethylene diamine

Betaine

Phosphoryl choline

β-Methoxy ethylamine

Carbamyl choline chloride

Urethane of β methyl choline chloride

Sarcosine

Carnitine

Methionine

Creatine

substitution of a phenyl group on the nitrogen resulted in a compound which could not be utilized by this organism.

Substitution on the first carbon atom produced compounds of varied activity. When one of the hydrogens was replaced by an ethyl group a compound much more active than ethanolamine was produced. The same compound with the amino group replaced by a nitro group could not be used at all, indicating that the organism was not able to reduce the nitro group. A further substitution of a methyl alcohol group on this first carbon atom resulted in a compound almost as active as choline. When the ethyl group was replaced with a methyl group the activity dropped to that of ethanolamine. Compounds derived by the substitution of two methyl or two hydroxylmethyl groups on this first carbon atom supported little or no growth. What is the significance of this inactivating influence exerted by the methyl group? The presence of the -COOH group in serine made this compound completely inactive. The organism apparently could not decarboxylate it.

Compounds in which the second carbon atom was substituted (Table 41) continued to demonstrate how unadaptive this organism was. It could not oxidize ethylamine or reduce glycine or sarcosine. It could not hydrolyze the ester and ether linkages through the -OH group. Apparently the activity of choline and ethanolamine depend upon a free -OH group since substitution of or through this group resulted in inactivation of the molecules. The slight activity of the acetyl compounds was probably due to their hydrolysis in the alkaline medium. The high activity of the two homologues γ-diethylaminopropanol and 2,3-propanediol-1-diethylamine was interesting as well as surprising. The difference in the activites of the two dihydrozy compounds No. 33 and No. 34 was unaccountable.

From this study of the structural specificity of choline for the growth of a Type III Pneumococcus, it may be concluded that the essential part of the choline molecule was the N-C-C-OH moiety. Substitution of the hydroxyl inactivated the compound. The only naturally occurring compound tested which could replace choline as a growth factor for this organism was ethanolamine.

The activities of these different choline and ethanolamine derivatives suggested certain facts about the biological function of choline in the Pneumococcus. Before discussing these facts, a brief review of the present knowledge of the function of choline in animal nutrition is in order.

Choline has been shown to prevent perosis in chicks (69), to act as a growth essential for chicks (70) and puppies (71), to enable growth of rats on a homocystine diet (72), to prevent fatty infiltration of the liver in rats (73), and in depancreatized dogs (74), to prevent renal hemorrhagic degeneration (75) and brain hemorrhages (76) in young rats, in addition to other less studied effects (77).

The knowledge of <u>how</u> choline prevents these abnormalities is progressing but is far from complete. The relation of choline to the fatty liver is probably through the formation of choline phospholipids which in turn function in the transport of fatty acids. Chaikoff (78), utilizing radioactive phosphorus has observed that choline accelerates the formation and removal of phospholipids in rat livers.

A satisfactory explanation of the function of choline in the prevention of renal hemorrhagic degeneration in rats, perosis in chicks and the promotion of growth in chicks and puppies has no acceptable experimental basis yet; but it seems probable that the two choline phospholipids are involved since both may be important in cellular organization as well as in lipid metabolism.

The function of choline in the growth of rats on a homocystine diet lacking in methionine has been shown to be due to its action as a source of labile methyl groups in the synthesis of methionine from homocysteine (72). The interrelationships between choline and other methylated compounds have been discussed by du Vigneaud (79). Evidence for the transfer of methyl groups from choline to homocysteine has been obtained by the use of isotopes. The transfer of methyl groups to guanidoacetic acid to form creatine (80) and to ethanolamine to form choline has also been demonstrated (81).

The relation of the structural specificity of choline to some of the known effects of choline has been summarized by

Moyer and du Vigneaud (82). Since many of the compounds included in this summary have been tested for their activity in promoting growth of Pneumococcus, a part of the summary is shown in Table 46.

After comparing the activity of these compounds on the growth of the Pneumococcus with some of their other known effects one may come to the following conclusions of the role of choline in pneumococcal nutrition.

(1) Choline does not function as a source of labile methyl group.s

(2) Choline is not essential because of its role in the biological synthesis of methionine and creatine.

(3) Choline may be synthesized from ethanolamine.

(4) This Type III Pneumococcus cannot synthesize ethanolamine from glycine or serine.

(5) Choline and ethanolamine may function as constituents of lecithin, sphingomylin and cephalin.

(6) That choline may have another function than the formation of a phospholipide is suggested by the inability of the organism to use phosphorylcholine.

(7) Choline does not act as a precusor of acetylcholine.

Table 46

Comparison of the Biological Effects of Choline Derivatives

Compound	Prevent Perosis	Fowl Growth	Growth with Homocysteine	Prevent Kidney Hemorrhages.	Liver Lipotropic	Growth Type III CHA Pn.
Aminoethanol	-(83)	-(83)	-(72)		-(73)	+
Betaine	-(70)	-(70)	+(72)	+(84)	+(73)	-
Ca phosphorylcholine			+(82)		+(85)	-
Choline chloride	+(70)	+(70)	+(72)	+(86)	+(87)	+
Choline methyl ether					-(88)	-
Creatine	-(83)	±(83)	-(72)		-(88)	-
Diethylmethyl-, dihydroxypropyl-ammonium chloride			-(72)			±
α,α-Dimethylcholine	-(89)	-(89)	-(82)			-
Dimethylethylcholine	+(89)	+(89)	+(82)	+(84)	+(82)	+
Homocholine			-(82)	+(90)		+
Methionine	-(83)	-(83)		+(91)	+(92)	-
β-Methyl choline	±(83)	-(83)	-(93)	+(82)	-(85)	+
Sarcosine			-(72)		-(94)	-
Tetraethanolamine					-(88)	+
Triethylcholine	-(95)	-(95)	-(72)	+(84)	+(96)	+

Discussion

A medium of essentially known composition has been described for a type III (CHA) Pneumococcus. This Pneumococcus survives, multiplies and maintains its virulence and type specificity when grown on a mixture of hydrolyzed casein, cystine, nicotinic acid, pantothenic acid, biotin, choline, certain inorganic salts, glucose and a reducing agent. With the exception of biotin, the nutritional requirements of this Type III Pneumococcus resembles that described by Rane (24) for Types I, II, V, and VIII. Rane has since found biotin to be required by all strains and types tested (40). That there are distinct qualitative and quantitative differences in the growth requirements of the pneumococcal types has been indicated by both Rane and Anderson (42). These differences are to be expected when one considers the basis of the differentiation of pneumococcal types.

A picture of the physical and chemical nature of the pneumococcal cell has been drawn by White (25 p. 297-299) and is recorded in part here. The basic protoplasm would contain a nucleoprotein with more or less constant composition for all pneumococci regardless of serological type. Another constituent of the protoplasm would be lipids, probably combined with the protein and conceivably loosely linked to the carbohydrate. Then the substance of the cell would also include a polysaccharide containing phosphorus and nitrogen and would, like the nucleoprotein, be possessed by every

Pneumococcus, whether of full virulence or degraded below the stage of any type identity. There would naturally be inorganic salts, but since little is known about them their actual composition cannot be defined. In this viable, watery solution of colloids and crystalloids there would be enzymes - proteases, lipases, invertases and saccharases - active in the metabolic processes of the cell and potentially able to destroy it. This globular mass of protoplasm would be surrounded by a capsule, largely carbohydrate in nature. The capsular material would be present when the cell was living in a favorable environment, in greatest amount when the surroundings were ideal, but entirely lacking when the cell had suffered from severe degenerative processes. The polysaccharides of these capsules would serve to distinguish the organism from many of the other members of the species and would differ in their content of nitrogen, of phosphorus and possibly of other elements. On hydrolysis the polysaccharides would yield unequal amounts and various kinds of cleavage products such as glucosamines, sugar acids, and the simpler sugar. The capsular polysaccharides would be linked to some of the protein, or even to the lipids, in a combination susceptible to cleavage by the enzymes of the cells, to the disruptive effects of physical forces, to the lytic action of bile, or to the hydrolyzing action of chemical agents.

In the construction of this complex functional pneumococcal cell what part is played by the substances required for growth? This picture is much more incomplete but partly from experimental data and partly from analogy some idea is obtained of the role of essential growth substances. The amino acids may provide essential structural units necessary for the construction of cell constituents. The inorganic salts may provide an osmotic effect (30) as well as essential elements needed in metabolic processes (31). It is possible that choline and ethanolamine too go to form some constituent of the cell structure. Whether this constituent is a phospholipid has not been shown. Choline containing phospholipids have been isolated from the tubercle bacillus (97), lactobacillus (98) and beer yeast (99). The role of biotin as a catalyst in intermediary metabolism has been suggested (100). It has been shown in yeast grown at low biotin levels, that the addition of biotin increases both anaerobic and aerobic fermentation rates immediately. Corresponding increases in respiration occur after about an hour and growth and turbidity increases after two hours. That biotin plays a similar role in pneumococcal metabolism is probable. There are some facts which suggest that pantothenic acid may be concerned with carbohydrate metabolism but they are by no means certain. The essential nature of nicotinic acid and its amide has generally been attributed to the needs of these compounds for syntheses of diphosphopyridine mucleotide or triphosphopyridine nucleotide, coenzymes in the intermediary metabolism of glucose (102). Recently the experiments of Saunders, Dorfman and Koser (103) and others by McIlwain (104) suggest that nitotinamide may function directly as a respiratory stimulator without being synthesized to a known coenzyme. The glucose provides the source of energy and ascorbic acid a favorable potential required for the proper functioning of the cell. The stimulating action of creatine, uracil, thiamine and glutamine is so small that no attempt is made to suggest a mode of action.

This very vague picture only serves to indicate the lack of fundamental knowledge of the biochemistry of the Pneumococcus. However, a great stride has been taken when the complex media required for growth has been replaced by a simpler medium of essentially known composition.

From a beginner's point of view it is interesting to speculate upon the lines of pneumococcal investigation which might benefit by this development. To mention only a few, the isolation and identification of cellular constituents including the much investigated capsular polysaccharides; the study of the enzyme systems; effect of suboptimal concentrations of the growth factors on the virulence, capsule formation, soluble specific substance and dissociation of Pneumococcus; action of growth inhibitors, particularly the sulfonamides.

The first task is to render the medium completely synthetic and to determine its ability to support the growth of other strains and types of Pneumococcus.

Summary and Conclusions

1. A semi-synthetic medium containing a casein acid hydrolysate, cystine, KH_2PO_4, $MgSO_4$, dextrose, ascorbic acid, nicotinic acid, pantothenic acid, biotin, choline, creatine, uracil, thiamine and glutamine has been developed for a Type III Pneumococcus.
2. No improvement in the rate or extent of growth was obtained by the addition of adenine sulfate, adenylic acid, asparagine, β-alanine, carnitine, folic acid, glucosamine, glutathione, glutamic acid, guanosine, inosotol, methionine, nicotinamide, p-aminobenzoic acid, pimelic acid, phthiocol, pyrodoxine, riboflavin, sarcosine, tryptophane and urea.
3. Within limited concentrations of choline, biotin, nicotinic acid and pantothenic acid the growth was proportional to the amount of each of the growth factors present.
4. The minimum effective concentrations of these accessory growth substances per ml. of medium were found to be 5.0 micrograms of choline, 0.0004 micrograms biotin, 0.4 microgram nicotinic acid and 0.2 microgram pantothenic acid.
5. It has been shown that this Type III Pneumococcus could not synthesize pantothenic acid from β-alanine or biotin from pimelic acid; but it could utilize nicotinamide in place of nicotinic acid.
5. Ethanolamine in ten times the molar concentration of choline could replace choline. In the presence of ethanolamine the organisms grew in long chains.

7. Experimental evidence for the multiplication, viability and maintenance of virulence and type specificity of the organisms in this medium has been presented.
8. The relation of the growth and death of the organism to the turbidity and acid production has been studied and discussed.
9. The specificity of choline has been tested with some 35 choline and ethanolamine derivatives.
10. From this study of the choline derivatives, the N-C-C-OH moiety of the choline molecule has been shown to be the active part.
11. The relation of the function of choline as a growth factor for Pneumococcus as compared to its other known biological effects has been discussed.

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