

STUDIES ON THE NUTRITION OF
CLOSTRIDIUM TETANI IN RELATION
TO TETANUS TOXIN PRODUCTION

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
James R. Howenstine
1962



This is to certify that the
thesis entitled
**Studies on the Nutrition of Clostridium
tetani in Relation to Tetanus Toxin
Production**

presented by

James R. Howenstine

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology and
Public Health

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REPORT OF

STUDIES ON THE NUTRITION OF CLOSTRIDIUM TETRAE IN RELATION TO TETANUS TOXIN PRODUCTION

by James H. Hovavetina

Studies in the nutrition of Clostridium tetani with reference to the production of tetanus toxin have been hampered by the absence of a toxin production culture medium in which all the ingredients were chemically defined compounds. The goal of this study was to develop a synthetic medium which would support both the growth of the organism and toxin production. Two approaches were taken in order to reach the goal.

In the first approach a complex medium which supported growth of the organism with high yields of tetanus toxin was standardized. The two complex ingredients in the medium were beef heart broth and M2 casein. The beef heart broth was standardized by purification through a dialysis procedure and drying for use in known amounts in appropriate amounts. A standard source of M2 casein was used and the purification attempts to purify it adequately failed. Quantitative determinations by chromatographic procedures indicated the extent to which each of the 16 amino acids existed in combined form in the M2 casein. Changes in the concentrations of three amino acids that occurred in the medium after toxin was produced were determined. The amino acids decreased in concentration while there was an increase in histidine and lysine content. From this information a semi-synthetic medium was devised which supported growth of the organism. The medium

yield of toxin at appropriate times in the growth of the culture was determined using an appropriate concentration of penicillin as a lysing agent.

In the usual approach a synthetic medium was utilized until it supported the growth of the fungus with the production of case toxin. A significant finding was that peptidases are not required for toxin production.

¹ME case is a pancreatic digest of casein produced by Sheffield Foods Co., Norwich, New York.

STUDIES ON THE NUTRITION OF CLOSTRIDIUM TETANI
IN RELATION TO TETANUS TOXIN PRODUCTION

By
James R. Howenstine

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1962

ACKNOWLEDGMENTS

The author wishes to express sincere appreciation to Dr. Charles L. San Clemente for his patience, encouragement, interest and guidance during the course of this study.

Furthermore, the author is indebted to his many associates at the Michigan Department of Health for their technical assistance and interest in the study. The author wishes to acknowledge particularly the helpful criticism and guidance rendered him by Dr. Russell Y. Gottshall for many stimulating discussions during the course of the study.

The author also wishes to acknowledge the support of both the Michigan Department of Health and the National Institutes of Health (Grant E-2088) for the funds and facilities which were made available for carrying out the study.

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GLOSSARY

Lf.--Flocculating unit of toxin. The amount of toxin which flocculates most rapidly with one unit of antitoxin in a series of mixtures containing varying amounts of toxin and constant amounts of antitoxin.

MLD.--Minimal lethal dose. The smallest amount of toxin which causes death within four days in guinea pigs weighing 315-385 g or mice weighing 18-20 g.

NZ case.--Pancreatic Digest of casein produced by Sheffield Farms Co., Norwich, New York.

S₁--First synthetic medium formulated which supported the growth of Clostridium tetani. The culture was incubated using anaerobic apparatus. See Table 21 for composition of S₁.

S₂--S₁ medium supplemented with 50 mg sodium thioglycollate.

STE.--Sterility test broth. Composition same as T medium with agar and resazurin omitted.

T medium.--Thioglycollate seed medium. Composition is given in Table 23.

T.D.--Test dose. The amount of toxin which when combined with 0.1 unit of standard antitoxin will result in the death of a 315-385 g guinea pig in 3½ to 4 days.

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I. INTRODUCTION

Tetanus toxin is important medically, since it is the agent which produces the symptoms of "lockjaw" in man and animals. Two ways in which its production is important are: (i) as an agent for the hyperimmunization of horses in the production of tetanus antitoxin and (ii) as a detoxified agent for the immunization of man and animals. In the production of tetanus toxin for these purposes the chief problem concerns the variability of toxin yields. Progress in the study of the causes of variability in toxin yields has been slow because toxin production was dependent on a complex culture medium containing unknown and variable chemical components.

The chief goal of this study was to develop a synthetic medium which would simultaneously support growth and toxin production. Factors of importance in achieving this goal included not only the determination of essential nutrients, but maintenance of adequate redoxing conditions in the medium, stimulation of toxin release from the cells at any time, and the recovery of toxin without denaturation during filtration of the culture.

Two general approaches were taken in order to obtain this goal. In the first instance a complex medium was standardized and chemically analyzed. This approach led to the development of a semi-synthetic medium. The essence of the second plan was to start with a synthetic medium and modify it until growth with toxin production was obtained.

II. LITERATURE REVIEW

Certain historical events have motivated the study of the extraction of the tetanus bacillus with respect to toxin production. Following the isolation of pure cultures of the tetanus bacillus by Kitasato (1889) and the production of the disease by Fieber (1890) using culture filtrates, Von Behring and Kitasato (1890) demonstrated the protective, antitoxic action of sera of animals which were inoculated with toxin. Descombey (1924) detoxified tetanus toxin by treatment with formalin and the protective value of the toxoid product was well demonstrated in humans on the battlefield of World War II.

There are a number of reviews on the subject of tetanus. An early one on tetanus work was that of Auglin and Kory (1902). Fildes et al. (1929) reviewed very extensively the information which was available at that time. More recent reviews are those by Pillemer and Robbins (1949), von Hayniger (1950, 1953), Ogilby (1954, 1955), Wright (1955, 1959), Fedinec and Natchik (1958), and those in the Mayo Clinic proceedings (Enter et al., 1957). Most of the literature on the subject pertains to the medical aspects of tetanus and appears in the current medical journals.

The outstanding achievement in obtaining fundamental knowledge of tetanus during this century was the purification, crystallization, and characterization of tetanus toxin by Pillemer and co-workers (1946, 1948, 1949). The crude toxin which was obtained using the medium of Mueller and Miller (1945) was crystallized by a fractional dialysis procedure at low

temperatures by controlling pH, ionic strength, and protein concentration. The crystalline toxin contained 15.7% nitrogen, 0.06% phosphorus and 1.04% sulfur. It had an isoelectric point of 5.1 and a sedimentation constant of 4.5 Svedberg units. It had a purity of 66×10^6 mouse MLD/mg N and its amino acid composition, determined microbiologically, consisted of 13 amino acids. Its minimum molecular weight was about 68,000. The protein nature of the toxin has since been confirmed in two other laboratories (Turpin and Raynaud, 1959; Largier, 1956) and no difference was found between the toxins produced from two strains of Clostridium tetani which differed both morphologically and physiologically.

Progress in the study of the nutrition of C. tetani in relation to the production of tetanus toxin has been hampered by the failure to produce it except on a chemically undefined medium. Also the toxin is relatively unstable (Pillener et al., 1946) and very difficult to assay. Manufacturers of tetanus toxin must take special precautions to avoid both the loss of the strain as well as the mutation of the organism to a non-toxigenic strain.

Most of the significant contributions which have been made during the last 22 years with reference to the nutrition of the organism and toxin production were made by the late Dr. J. H. Mueller and co-workers at Harvard (Mueller and Miller, 1940, 1942, 1943, 1945, 1947, 1954, 1955, 1956; Mueller et al., 1943; Feeney et al., 1943a, 1943b; Fisek et al., 1954; Miller, 1955a; Miller et al., 1956).

When World War II was about to start there was an intensified demand for large supplies of tetanus toxoid for immunization purposes. The peptone used in domestic media at that time was blamed for numerous allergic reactions. Noting the similarity in the problems of tetanus and diphtheria

toxin production, Dr. Mueller tried to solve the problem by replacing the peptone with an acid hydrolyzate of casein. Unfortunately the yields of toxin were unsatisfactory and he failed to prepare a truly synthetic medium for its production.

He then studied the use of enzymatic digests of casein since the enzymatic hydrolyzates of peptones which were used in Europe at the time were not allergenic. Using a trial and error approach he and his co-workers developed a good toxin production medium (Mueller and Miller, 1954). It contained a pancreatic digest of casein and beef heart infusion. Both of these materials contained unidentified inhibitory substances. Important observations during the course of this study were: (i) the culture in this medium became iron tolerant, non-spore forming, and produced H_2S during its growth which was found to be inhibitory to toxin formation; (ii) stock vitamin solutions had to be stored separately; (iii) the seed culture was incubated anaerobically but the production culture was incubated aerobically; and (iv) after sterilization, the medium had to be cooled rapidly.

Of current nutritional importance were the reports from Mueller's laboratory (Mueller and Miller, 1956; Miller, 1955a) that histidine peptides played an essential role in toxin production.

Three novel developments in methods of toxin production have been reported in recent years. One involved the preparation of tetanus toxin by growing the organism in a cellophane bag immersed in the medium (Koch and Kaplan, 1953). The second development used a semi-continuous method (Thomson, 1957). Neither method has found wide application. The third development concerned the adaptation of the organism to growth in a nestless medium (Stone, 1953). This adapted strain might become the most popular strain for industrial use.

The general nutrition of C. tetani without special consideration for toxin production has been studied by various workers. Clifton (1942) concentrated on the utilization of amino acids and related compounds by the organism. Lerner and Mueller (1949) were especially interested in the utilization of glutamine. Lerner and Pickett (1945), Prevot and Boorsma (1941), Tasman and Pondman (1941) and Martinez and Rittenberg (1959) reported on glucose utilization by C. tetani. Kaufman and Humphries (1958) devised a chemically defined medium which they found would inconsistently support growth of C. tetani.

III. METHODS AND MATERIALS

A. Culture Maintenance

Two strains of Clostridium tetani were used in this study. The "Mueller" strain was obtained from the New York State Health Laboratories¹ in 1954. The EA-3 strain was obtained from the Massachusetts Public Health Biologic Laboratories² in 1959. The EA-3 strain was derived from the Mueller strain and differed from it by its ability to grow and produce tetanus toxin in a meatless medium. Spore formation in either strain has not been demonstrated.

Both strains were maintained in the frozen state and transferred after thawing at weekly or semimonthly intervals. The EA-3 strain was transferred daily into thioglycollate seed medium. The Mueller strain was subcultured into veal infusion seed medium and incubated anaerobically (Rosenthal, 1937) at 35 to 36 C for 18 to 24 hours. Cultures were checked for contamination by microscopic examination of Gram stained smears.

B. Growth Measurement

Growth of the culture was measured by serial tube dilution in thioglycollate seed tube medium or by optical density at 530 mμ against distilled water using a Coleman Junior spectrophotometer, model 6A³. In the latter method 1.0 ml of the culture was first acidified with 0.2 ml concentrated HCl to dissolve the iron sulfide which formed during growth.

¹New York State Health Laboratories, Albany 1, N. Y.

²Massachusetts Public Health Biologic Laboratories, Boston 39, Mass.

³Coleman Instrument Company, Maywood, Ill.

C. Assays of Tetanus Toxin

Strict adherence to a standard technique was required to obtain reliable and reproducible toxicity values. During the study Seitz filters were replaced by sintered glass filters for the removal of the bacterial cells from the toxic culture medium. Aseptic technique was followed as far as possible in carrying out the assays on the toxic filtrates. Dilutions of the culture filtrate were made in Connaught diluent (Moloney and Taylor, 1932) using calibrated pipets. The fluid was drawn up to the "0" mark on the pipet and the contents of the tube mixed by rinsing the pipet several times prior to the transfer of a portion of the fluid. Care was taken to avoid aeration in the mixing of the fluids so that denaturation of the toxin was minimized. A fresh pipet was used to make each dilution of the fluid and ten final dilutions were usually made for each MLD¹ test. One milliliter of each final dilution of the filtrate was injected subcutaneously into the abdomen region of either one 315-365 g guinea pig or into each of two 18-20 g mice. The injections were made as soon as possible after the filtrate dilutions were prepared and care was taken not to expose the test solutions to sunlight.

Five to ten guinea pigs were used for T. D.² tests. This test was carried out by adding 2.0 ml of diluted toxin to 2.0 ml of standard antitoxin which had been diluted to contain 0.1 unit per 1.0 ml. After incubation in the dark for one hour, 2.0 ml of the mixture were injected subcutaneously into the ventral region of the guinea pig. The end point was the highest dilution of toxin which caused death in 3½ to 4 days in one of the animals.

¹MLD = minimal lethal dose.

²T.D. = test dose.

In the in vivo assays the animals were observed twice a day for death and daily for symptoms of tetanus intoxication. The severity of toxicity, determined by visual inspection and handling of the animals, was graded from one plus to four plus. Toxicity was characterized by increased muscle tonus, curvature of the spine, asymmetry of body outline when the resting animal was viewed from above, stiffness or paralysis of neck or legs, and inability to rise from a smooth, flat surface when the animal was placed on its side.

The culture filtrate was assayed by the in vitro flocculation method recommended by the National Institutes of Health (1953).

D. Amino Acid Analysis

The procedures of Moore and Stein (1948, 1951, 1954) and Moore et al. (1958) were used for the quantitative determinations of amino acids. Any variation from these procedures are discussed in the Experimental section.

Samples which were obtained by elution of the material from a resin column with a buffer were desalted using the resin column procedure of Rinker (1957) prior to identification by paper chromatography.

Except where indicated in the Experimental section, two dimensional $18\frac{1}{2} \times 22\frac{1}{2}$ inch Whatman No. 1 filter paper chromatograms were prepared in an R. S. Co.¹ A-300 chromatograph. The chromatograms were developed first in a solvent having a ratio composition of 4 ml liquid phenol²:1 ml distilled water:2 ml 8-hydroxy-quinoline. For the second dimension a solvent having a ratio composition of 375 ml butanol:90 ml acetic acid:375 ml distilled water was used. The location of the compounds on the paper was determined using

¹Research Specialties Corp., Richmond, Calif.

²Gannett Stephens Co., Lansing, Mich.

a spray reagent composed of 200 mg ninhydrin in 100 ml acetone and a heat treatment of about two minutes at about 90 C. The identity of the compounds was based primarily on the relative positions of known and unknown compounds which were chromatographed at the same time under identical conditions.

IV. EXPERIMENTAL

A. Variability of Toxin Yields with a Medium Formulated by Mueller and Miller

In the large scale production of tetanus toxoid, the medium used at the Michigan Department of Health and widely throughout the world for the cultivation of the tetanus organism is the one proposed in 1954 by Mueller and Miller. The formula for this medium is shown in Table 1.

The problem of variability in yields of toxin from this medium is well known to commercial producers of tetanus toxoid. The data given in Table 2 illustrate this variation in the potency of eight consecutive large scale lots of tetanus toxin.

In the production of these lots, six, thirty-five liter stainless steel kettles were used for each. Such results are typical of lots produced either in stainless steel kettles or in pyrex animal jars.

B. Standardization of the Medium Formulated by Mueller and Miller

1. Use of cellophane bag

Mueller and Miller's formula contained two components of unknown and variable composition which could cause significant variations in the yields of toxin. Since it was desired to study the metabolism of Clostridium tetani in relation to tetanus toxin production, the first goal of the study was to develop a standard method of cultivation.

The relatively recent innovation of growing the organism in a cellophane bag (Figure 1) was found to be undesirable because of easy contamination and volume changes.

Table 1. The median formula of Muller and Miller (1954) for tetanus toxin production.

Component(s)	Quantity
KCl base*	22.5 g
Deaf Heart Infusion**	50 ml
Glucose	11 g
KaCl	2.5 g
K ₂ HPO ₄	2 g
KH ₂ PO ₄	0.15 g
H ₂ PO ₄ ·7H ₂ O	0.15 g
Cystine	0.25 g
Tyrosine	0.5 g
3- Parathionate***	1 ml
Ureacl	0.5 ml
Fluclon	0.25 ml
Riboflavin	0.25 mg
Pyridoxine	0.25 mg
Biotin	0.5 mg
Reduced Iron****	0.5 g
Water, Distilled <u>1</u>	1000 ml

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Table 2. Potencies of eight large scale lots of tetanus toxin produced in the medium of Mueller and Miller (1954)

Lot	MLD*/ml	T.D.*/ml	Lf*/ml	ml harvested
1	300,000	250	44	145,800
2	450,000	300	60	123,000
3	150,000	200	41	145,000
4	150,000	300	45	148,000
5	350,000	250	47	123,100
6	200,000	350	47	127,500
7	300,000	300	47	150,300
8	300,000	300	45	149,900

*MLD = minimal lethal dose; T.D. = test dose; Lf = flocculating unit of toxin.

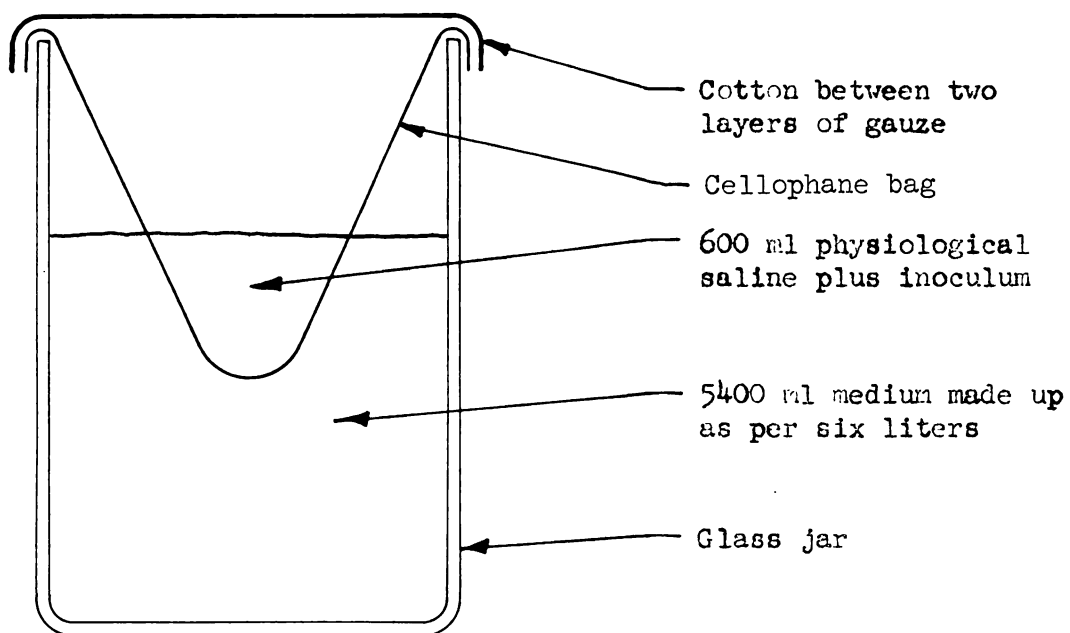


Fig. 1. Dialysis apparatus using a cellophane bag.

2. Use of cellophane tubing

Another method (Figure 2) using cellophane tubing instead of a sheet of cellophane gave high MID values without contamination but the volume of the toxin inside the tubing decreased from 680 ml to 156 ml during a 12-day incubation period. The difficulty of measuring volume changes in such an arrangement without disturbing the culture remained a problem.

3. Use of predialyzed medium

Since the organism could grow and produce good yields of toxin using only dialyzable materials, the possibility of growing the organism in predialyzed medium was investigated. This would eliminate the errors of volume changes during the growth of the organism. When the medium (complete except for sugar and vitamins) was dialyzed at 0-5 C at a sixfold concentration against an equal volume of physiological saline which was exchanged for fresh saline the following day, inefficient dialysis resulted unless the bag was rotated at a slow speed. Mouse MID values of 400,000 to 600,000 per ml were obtained on the culture filtrates when the organism was grown in predialyzed medium but because the rotating cellophane bags ruptured easily, it was decided to dialyze only the two complex components in the medium--NZ case¹ and beef heart infusion.

4. Standardization and amino acid composition of NZ case

a. Fractional dialysis and testing of fractions

Craig and King (1955) were able to fractionate complex mixtures of amino acids and peptides by dialysis. Results of preliminary experiments indicated that NZ case could be fractionated using this principle and that the dialysis of a 6% NZ case solution gave the greatest dry weight yields

¹Pancreatic digest of casein produced by Sheffield Farms Co.,
Norwich, N. Y.

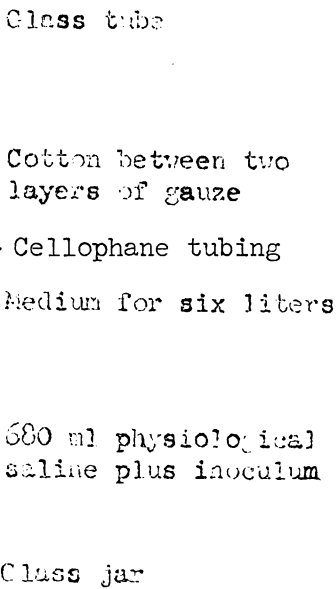


Fig. 2. Dialysis apparatus using cellophane tubing.

of the various fractions. This was determined in an experiment carried out at 30 C using 1500 ml of 1%, 2%, 4%, and 6% HE case solutions in cellophane tubing immersed in 4000 ml of 1.1 N acetic acid solutions in each of four, 923 X 100 mm pyrex tubes. HE case precipitated at a concentration of greater than 6%. A manifold was constructed for equalizing mixing of the contents of the cellophane tubes by aeration. The rate of aeration was 50-100 bubbles of cotton-filtered air per minute.

Table 3 shows that essentially the same percent of HE case passed through the cellophane tubing at any time interval during dialysis of any of the HE case solutions tested. These results were calculated from duplicate, dry weight determinations at various intervals.

HE case was then fractionated by this procedure into four fairly large fractions starting with a 6% HE case solution. The dialysis times of these fractions were: 0-12 hours, 12-24 hours, 0-24 hours, and 0-65½ hours. The percent of HE case which had passed through the cellophane tubing for each of the fractions can be determined from the curve plotted in Figure 3. The material in each of the fractions was dried from the frozen state to give a white, hygroscopic material which was neutral to hydric paper. None of the fractions contained material which precipitated upon the addition of 5% trichloroacetic acid.

Each of these fractions was compared with unfractionated HE case for toxin production using 75 ml of medium in each culture tube. Ten pyrex culture tubes, 25 X 325 mm, were used per variable. The toxin was harvested by filtration of the culture after incubation for six days at 35 C followed by six days at 0-4 C. Less than 50,000 mouse LD₅₀/ml were obtained on the culture filtrates from media which had contained fractions of HE case but from two to four times this value was obtained when the culture medium

Table 3. Amount dialyzed of different concentrations
of NZ case at various intervals

% NZ case initially	% NZ case dialyzed					
	Dialysis time (hr)					
	4	8	18	24	29	47
1	25.4	37.8	54.8	58.7	61.9	66.3
2	24.4	37.1	54.6	60.5	62.2	69.6
4	25.4	38.7	54.3	61.6	63.0	68.7
6	23.7	39.1	56.0	62.2	63.2	69.8

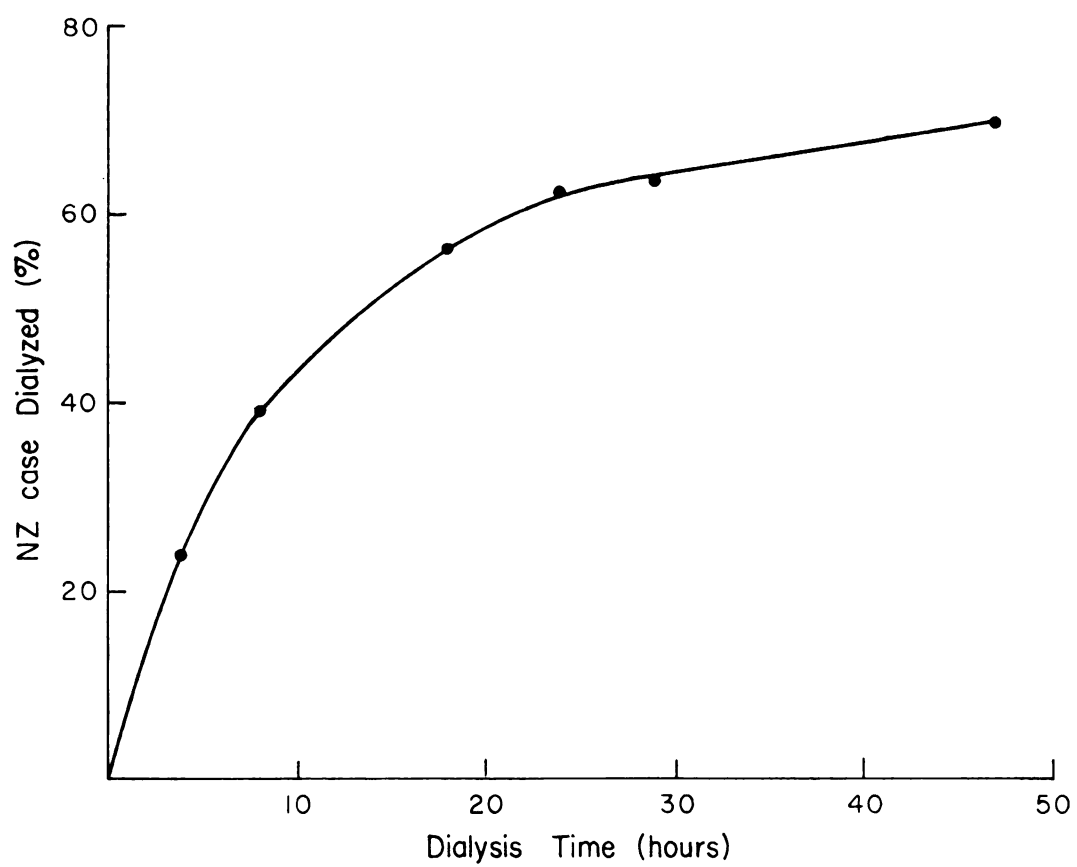


Fig. 3. The rate of dialysis of a 6% NZ case solution in a 0.1 N acetic acid system.

contained unfractionated NZ case. From these tests it was concluded that none of the NZ case fractions would substitute effectively for unfractionated NZ case in the medium for toxin production.

One lot of NZ case was set aside as a standard ingredient for future experiments as a result of the knowledge gained from this experiment and from experiments in which two lots of NZ case were compared for the production of toxin. Table 4 shows that a certain lot of NZ case may stimulate greater yields of toxin than some other lot (lot 00F3). Lot 70D13 was the lot chosen as a standard NZ case source.

b. Amino acid composition of NZ case

Since NZ case is a powdered tryptic digest of casein, it was expected to contain a complex mixture of amino acids and peptides. The manufacturer has stated that a typical lot of NZ case would have a total nitrogen value of about 12.7% and an amino nitrogen value of about 5.1%.

Only one group of investigators has tried to fractionate NZ case for studies in the production of tetanus toxin. This group, under the leadership of Dr. J. H. Mueller at Harvard University, was able to fractionate NZ case into neutral, acidic, and basic portions which were found to be very complex by autoradiographic studies. Not even a qualitative analysis of the material was achieved.

After much consultation and a review of the literature for a procedure which could be used quantitatively for the determination of amino acids and small molecular weight peptides in the medium, it was decided to choose the procedure of Moore and Stein (1951, 1954) and Moore et al. (1958) using ion exchange resin

Table 4. Variability in the yield of tetanus toxin
from two lots of NZ case

Experiment	NZ case lot	Liters of toxin harvested	MLD*/ml	T.D.*/ml
1	70D13	20.2	350,000	300
1	00F3	20.8	100,000	<100
2	70D13	8.7	400,000	300
2	00F3	21.5	150,000	150

*MLD = minimal lethal dose; T.D. = test dose.

columns together with appropriate modifications. These workers have stated that their procedures could determine all the common amino acids and peptides up to ten units in size. These procedures are based on the principle of adsorption and continuous sequential elution of compounds from the resin with appropriate buffers and rigid control of pH, ionic strength, and temperature. The eluted materials were collected in fractions and quantitatively assayed by a carefully controlled reaction with a ninhydrin reagent to give a spectrum of ninhydrin positive compounds. The details of the procedures which were used here were essentially the same as those published by the authors except as indicated.

The first of the procedures to be investigated was the one (Moore and Stein, 1954) using a 150 X 0.9 cm column, Dowex 50-X4 resin, and an eluent of continuously changing pH and ionic strength. Although several months were spent using this procedure to analyze known samples, adequate control of the mixing of the buffers to give a gradient elution of the compounds was not achieved and this procedure was discontinued in favor of the one which was published more recently (Moore et al., 1958).

In this investigation, Amberlite IR-120, Type III was fractionated into five parts by the hydraulic fractionation method of Hamilton (1958) and fraction D (particle diameter of approximately 56 μ) was used for the 150 cm column for chromatographic analysis of neutral and acidic compounds. Fraction C (particle diameter of approximately 40 μ) was used for the 50 cm column for chromatographic analysis of the basic compounds. The samples were collected in a model 1205, R. B. Co., automatic fraction collector equipped with a 1.0 ml volumetric siphon unit. Six hundred samples were collected in runs completed with either of the basic columns. From each

sample, 0.5 ml was pipetted into a matched set of photometer tubes and the ninhydrin color developed according to the procedure of Moore and Stein (1948) with modifications as suggested by Dr. Selma L. Bandemer, Biochemistry Department, Michigan State University.

In the standardization of the procedure color development of known amino acids was studied with the modified ninhydrin reagent under the same conditions as those established with the eluates. The modified ninhydrin reagent was prepared fresh each day. The hydrindantin which was used as an antioxidant in the reagent was purchased from Nutritional Biochemicals Corporation¹. Standard curves were prepared for each of the amino acids found in preliminary column runs in the filtrates using 5, 10, or 15 ml of a 50:50 water-95% ethanol mixture as a diluent and plotting optical density units at the appropriate wavelength against different concentrations of amino acid. From these standard curves, it was possible to convert directly the total number of ninhydrin color units obtained in all the samples collected from any single peak (after correction of the values for the average base line value) to micromoles of amino acid. By the conversion of micromoles of amino acid to mg of amino acid, a direct relationship between optical density units and the weight of the amino acid was obtained as shown in Table 5.

It was found that the maximum number of determinations which could be carried out in one batch was 50, or the number normally possible within an hour. After one hour the color which develops through the reaction of ninhydrin with an amino acid may decrease as much as 1.8% per hour. An automatic pipetting machine was calibrated to dispense 5.0 cc of diluent per tube and this machine made it possible to do more tubes than could have been done in a single batch by hand. The soft glass

¹Nutritional Biochemicals Corporation, Cleveland, Ohio

Table 5. Reference table expressing optical density units equivalent to 0.01 mg of amino acid in three ranges

Amino acid	ml of diluent		
	5.0	10.0	15.0
Aspartic acid.	320	188	132
Threonine	328	193	139
Serine	430	252	176
Glutamic acid	314	187	132
Valine	363	209	145
Methionine	322	195	131
Isoleucine	359	206	149
Leucine	359	208	149
Tyrosine	254	146	105
Phenylalanine	267	158	106
Glycine	607	352	246
Alanine	691	405	287
Proline	109	69	43
Arginine*	274	134	111
Taurine	378	218	154
Histidine*	292	167	123
Lysine*	361	210	147

*Calculated as free base.

photometer tubes 150 K18 mm were calibrated according to Moore and Stein (1948) using a methyl red solution in 0.03 N HCl so that a reading of 0.655 ± 0.005 was obtained on the optical density scale when read at 525 mu against water set at zero. The sample tubes were read on the Coleman Junior spectrophotometer, model 6A, at 570 mu except for proline and hydroxyproline which were read at 440 mu. The blanks were read against a reference tube of the diluent. The blank tube with the middle value was chosen, and the instrument set at zero prior to reading the sample.

In the analysis of ME case for amino acid content, the ME case was hydrolyzed by the procedure recommended by Dr. Selma L. Bandemer. In this procedure 50 ml of 20% HCl were added to 2.5 g ME case in a 125 ml Erlenmeyer flask. The material was then heated in an autoclave at 15 lb pressure for six hours. The material was then evaporated to dryness on a steam bath. The residue was transferred to a 100 ml beaker with 50 ml distilled water and taken to dryness on a steam bath. The residue was resuspended once more in 50 ml distilled water and again taken to dryness on a steam bath. The residue thus obtained was transferred with distilled water to a 50 ml volumetric flask and diluted to the mark. This solution represented then, a hydrolyzate of 50 mg of ME case per 1.0 ml. Various procedures were tried using this solution before it was decided that the procedure of choice was to dilute the sample 1:10 and add 0.5 ml (or 2.5 mg ME case hydrolyzate) sample to the Amberlite resin column for analysis. Of each 1.0 ml sample collected, 0.5 ml was analyzed using 1.0 ml of the ninhydrin reagent.

Table 6 shows the results obtained from this procedure. It can be observed that there was 114.6% recovery of the HZ case components. Such recoveries are typical for protein hydrolyzates since one molecule of water is taken up for every dipeptide cleaved. The table also shows that the analysis accounts for a value of 16.1% of the total weight of HZ case as nitrogen. This value is reasonable and differs from the value given by the manufacturer for a "typical" HZ case lot by only 3.4%.

Where peaks were indicated from the analysis of half of each sample, the remainder of the samples of any one peak was pooled and the samples were desalted using the usual procedure as described in the section on methods. The desalted samples were then identified qualitatively by two-dimensional paper chromatography as previously described. Differentiation of the large number of amino acids was not achieved using any of several procedures involving one-dimensional paper strip chromatography, or a two-dimensional paper (7 3/4 x 7 3/4 inch) chromatography procedure. The Rf values were essentially the same as given by Block et al. (1955) but identification was based primarily on the relative positions of the unknowns and knowns which were processed simultaneously.

3. Purification, standardization, and composition of beef heart infusion

The requirement for beef heart infusion for maximum toxin yields was established in experiments performed prior to its purification. In one of these experiments (Table 7) it was also established that: (i) the yield of toxin decreased as the amount of glucose in the medium was reduced; (ii) the yield of toxin could usually be increased if the culture was incubated an additional four days at 4°C after the six day incubation at 37°C; and (iii) a good correlation existed between the extent of autolysis as observed microscopically, the quantity of trichloroacetic acid precipitable material in the culture filtrate, and the in vivo toxicity values obtained.

Table 6. Amino acid composition of NZ case by the chromatographic method of Moore et al. (1958)

Amino acid	Amino acid in NZ case	Nitrogen in amino acid	Nitrogen in NZ case
	mg/100 mg	mg/100 mg	mg/100 mg
Aspartic acid	5.60	10.52	0.590
Threonine	3.92	11.72	0.460
Serine	4.92	13.33	0.656
Glutamic acid	19.64	9.52	1.870
Proline	20.54	12.17	2.500
Glycine	1.48	18.66	0.276
Alanine	1.96	15.72	0.308
Valine	5.84	11.96	0.698
Methionine	1.56	9.39	0.147
Isoleucine	3.36	10.68	0.358
Leucine	8.68	10.68	0.925
Tyrosine	3.08	7.73	0.238
Phenylalanine	4.20	8.48	0.356
Arginine	10.64	32.16	3.420
Histidine	7.64	20.05	1.530
Lysine	11.56	15.34	1.772
TOTAL	114.6		16.114

Table 7. Effect of glucose and beef heart infusion on autolysis, soluble protein*, IID*, and LF*

Glucose	Beef heart infusion	House IID/ml		LF/ml		Soluble protein	Autolysis
		5 days	10 days (4 days @ 4 C)	5 days	10 days (4 days @ 4 C)	5 days	6 days
g/liter							
11	-	< 50,000	200,000	9	12	2+	2+
11	+	700,000	>1,000,000	12	27	4+	4+
8.25	+	300,000	700,000			4+	4+
5.5	+	<100,000	>100,000 <200,000			4+	4+
2.75	+	<1,000	>10,000 <100,000			2+	2+
1.1	+	<1,000	<10,000			-	+
0	+	1,000	1,000			-	±

*Soluble protein = material precipitated by trichloroacetic acid;
IID = minimal lethal dose; LF = flocculating unit of toxin.

Since a requirement for beef heart infusion had been established, the elimination of large molecular weight components of the material through the use of some dialysis procedure was attempted.

Preliminary experiments led to the development of a large scale dialysis procedure. Accordingly, fifty-five pounds of lean, ground beef heart were boiled for twenty-five minutes in 27.5 liters of distilled water. The filtrate of the cooled and defatted broth was divided equally into twenty-five cellophane tubes, each of which was immersed in 3,000 ml of distilled water within a 923 K 102 mm pyrex tube. The lower end of the cellophane tube was tied with rubber bands and string and the upper end was folded over the rim of the open end of the glass tube where it was secured by string. The glass tube was then covered with a layer of cotton and gauze which was also tied to the tube with string. The tubes were then autoclaved at 121 C for twenty minutes prior to incubation for 15 hours at 4 C. The dialyzates were pooled and dispensed into sixty ml serum bottles so that each bottle would contain material having a dry weight of 62.2 mg or the dry weight of crude beef heart which is required for 75 ml, the usual amount of culture medium in one tube. This standardized medium ingredient was dried from the frozen state in bottles. It was found to be a satisfactory substitute on a dry weight basis for the crude beef heart infusion used in the toxin production medium (Tables 8 and 9).

The beef heart infusion dialyzate before drying from the frozen state (i) contained no trichloroacetic acid precipitable protein; (ii) contained 5.6 mg dry weight per ml; (iii) contained 0.57 mg Kjeldahl N per ml; and (iv) contained about 0.4 mg reducing sugar per ml. Thus, one could estimate that on a dry weight basis, it contained about 7% reducing sugar.

Table 8. Comparison of crude to dialyzed beef heart infusion in toxin production

	MLD*/ml	Lf*/ml
Crude beef heart infusion**	1,100,000	45.1
Dialyzed beef heart infusion**	1,600,000	43.75
Crude beef heart broth***	2,000,000	54.9
Dialyzed beef heart broth***	2,500,000	54.9

*MLD = minimal lethal dose; Lf = flocculating unit of toxin.

**Harvested after 6 days at 36 C.

***Harvested after 6 days at 36 C plus 6 days at 4 C.

Table 9. Effect of beef heart preparations
upon tetanus toxin production

Material	MLD*
Beef heart broth dialyzate	500,000
Non-dialyzed beef heart broth	700,000
Crude beef heart broth	500,000

*MLD = minimal lethal dose.

Cultures incubated 6 days at 35 C plus 9 days at 0-4 C.

The frozen and dried beef heart infusion dialyzate was very hygroscopic, had the odor of caramel, was yellowish in color, and was very soluble in water. An amino acid measurement was conducted by diluting 62.4 mg or the contents of one frozen and dried sample of the beef heart broth dialyzate with 6.24 ml of 5% trichloroacetic acid and analyzing 0.5 ml, or 5 mg, of the sample. Table 10 shows the composition of the sample in terms of neutral and acidic compounds. In individual runs the peaks were identified when the sample was analyzed quantitatively by observing the increase in the peak size when the proper amino acid (0.2 mg taurine, 0.2 mg glutamic acid, 0.2 mg serine, 0.2 mg glycine, 0.3 mg urea, or 0.3 mg alanine) was added. The only peak in the analysis for basic compounds was one in the position corresponding to ammonia. About 12% of the purified beef heart material was accounted for in terms of neutral or acidic amino acids.

C. Determination of the Rate of Tonic Formation in the Standard Medium

Before the rate of tonic formation could be determined, knowledge of the maximum tonic yields at any given time during the growth of the organism in the standard medium was necessary. It was frequently noticed that when small volumes of the culture were filtered through asbestos filters, the toxicity of the filtrate was quite variable. If sintered glass filters were used to obtain the tonic, the toxicities (Table 11) of the filtrates were not only more constant but higher. Sintered glass filters were therefore routinely used hereafter.

Table 10. Identified amino acids contributed by purified
beef heart infusion to the toxin production medium

Amino acid	Concentration in purified beef heart infusion	Concentration in toxin medium
	mg/100 mg	mg/100 ml
Taurine	1.478	1.230
Serine	8.580	7.139
Glutamic acid	1.360	1.132
Alanine	<u>0.594</u>	<u>0.494</u>
TOTAL	12.012	9.995

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Table 11. Yield of toxin from the same culture
by two filtration methods

Type of filter	Mouse MLD*/ml
Seitz (asbestos)	100,000
Seitz (asbestos)	< 100,000
Seitz (asbestos)	$\leq$ 100,000
Sintered glass	1,000,000
Sintered glass	1,000,000
Sintered glass	1,000,000

*MLD = minimal lethal dose.

Also, since there was considerable evidence concerning the intracellular presence of toxin, a lytic technique was necessary to measure the total toxin prior to autolysis. Mir Chamsy and Sadegh (1958) had found that lysis of C. tetani with the liberation of toxin could be brought about using 1,000 units of penicillin per ml of culture. It was decided therefore to determine the effect of penicillin G on toxin production. The results of the treatment of cultures at different ages and densities with various concentrations of the antibiotic are shown in Table 12. The culture was incubated an additional twenty-four hours at 35°C after addition of the antibiotic and the toxin was then obtained by filtration through a sintered glass filter. As a result of these studies it was decided to use 10 units of penicillin G per ml of culture followed by one additional day of incubation and filtration through sintered glass prior to assay.

By using the penicillin treatment the rate of toxin formation in relation to the density of the culture could be calculated from the data presented in Table 13. The data also showed low toxin values were obtained without antibiotic treatment in early growth phases of the culture.

D. Studies on Amino Acid Utilization in Cultures of C. tetani

Table 14 shows a comparison of three analyses which were made on the same standardized toxin producing medium just prior to its inoculation with the tetanus organism. These analyses were performed by adding 2.1 ml of the medium to 0.35 ml of 3% trichloroacetic acid and analyzing 0.5 ml of the sample for nitrogen, positive or negative by the Moore and Stein procedure. The data obtained were then converted to $\mu\text{g}/100\text{ ml}$ of free amino acid in the original medium. The values obtained for each amino acid were within 1% of the average.

Table 12. Effect of different amounts of penicillin added at various intervals upon toxin production

Incubation time	Optical density* (530 mμ)	Average MLD**/ml value with units penicillin G/ml culture				
		0 unit	1 unit	10 units	100 units	1000 units
hr						
24	0.23	10	80,000	80,000	70,000	10,000
48	0.44	<100	300,000	300,000	100,000	100,000
72	0.47	50,000	300,000	300,000	300,000	200,000
96	0.36	200,000	200,000	200,000	200,000	200,000

*Optical density of the culture determined just prior to addition of penicillin G.

**MLD = minimal lethal dose.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220	1221	12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Table 13. Effect of an optimal amount of penicillin at various intervals upon toxin yield

Incubation time	Optical density* (530 mμ)	Average MLD**/ml	
		Penicillin G per ml culture	
		0 units	10 units
hr			
0	0.135		
17.5	0.239	100	50,000
41.5	0.43	10,000	200,000
65.5	0.538	600,000	700,000
89.5	0.368	700,000	400,000

*Optical density of the culture determined just prior to addition of penicillin G.

**MLD = minimal lethal dose.

Table 14. Quantitative analysis of the free amino acids
in the standard medium by the chromatographic technique

Amino acid*	Concentration of free amino acid per analysis			
	No. 1	No. 2	No. 3	Average
	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
Threonine	26	31	31	29
Serine	28	32	29	30
Glutamic acid	28	32	28	29
Valine	62	72	59	64
Methionine	34	32	29	32
Isoleucine	32	33	32	32
Leucine	136	139	128	134
Tyrosine	90	94	81	88
Phenylalanine	76	67	74	72
Lysine	116	129		123
Histidine	25	25		25
Arginine	71	55		63

*Cystine is not determined by this method.

An estimate of the percent of amino acid in peptide form in the medium was obtained (Table 15) by subtracting the sum of the individual free amino acids determined in the medium from the sum of the corresponding amino acids found in the NZ case acid hydrolyzate and the free amino acids added to the medium. Such an estimate ignored the contributions made to the medium by the beef heart broth dialyzate which contributed only 3.7% as much weight to the medium as NZ case.

The changes that occurred in the free amino acid composition of the medium as it was metabolized by the organism for various periods are shown in Table 16.

A study of the data in Tables 13 and 16 reveals that threonine, serine, and glutamic acid disappeared from the medium at the time that toxin was being produced rapidly. The basic amino acids, lysine and histidine, increased in concentration whereas the other amino acids were decreasing in concentration at this time. A number of other ninhydrin positive peaks appeared in the various spectra particularly after $65\frac{1}{2}$ hours when the cells were autolyzing. These ninhydrin positive materials were not identified.

B. Effect of Modifications of the Standardized Medium on Toxin Production

A comparison of the NZ case dialyzate fractions previously described to unfractionated NZ case in the standardized medium for toxin production was made using the penicillin treatment and sintered glass filters in the procedure. The results given in Table 17 reaffirmed the fact that none of the NZ case fractions could substitute effectively for unfractionated NZ case in the medium on the basis of the amount of toxin produced.

Table 15. Estimate of combined amino acids
in the standard medium

Amino acid*	Amino acid added directly to medium	Total amino acid in medium from NE case	Free amino acid in medium	Combined amino acid in medium	Portion of amino acid combined in medium
	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
Aspartic acid		126		126	100.0
Threonine		88	29	59	67.0
Serine		111	30	81	73.0
Glutamic acid		442	29	413	93.5
Proline		461		461	100.0
Glycine		33		33	100.0
Alanine		44		44	100.0
Valine		131	64	67	51.1
Methionine		35	32	3	8.6
Isoleucine		75	32	43	57.3
Leucine		195	134	61	31.2
Tyrosine	50	69	38	31	26.1
Phenylalanine		95	72	23	24.2
Histidine		172	25	147	85.5
Arginine		240	63	177	73.8
Lysine		260	123	137	52.7

*Cystine is not determined by this method.

Table 16. Amount of free amino acids remaining in culture filtrates at various intervals during growth of C. tetani

Amino acid	Amino acid concentration (mg/100 ml)					
	0 hr	17.5 hr	41.5 hr	65.5 hr	89.5 hr	113.5 hr
Threonine	29	0	0	0	4.8	5.1
Serine	30	0	0	0	3.1	5.7
Glutamic acid	29	3.7	0	0	14.1	15.9
Valine	64	34.2	33.2	33.6	35.3	41.3
Methionine	32	6.9	36.9	43.8	60.0	60.7
Isoleucine	32	23.3	24.0	36.5	41.6	48.2
Leucine	134	68.3	68.7	48.5	46.3	47.6
Tyrosine	88	41.1	22.8	14.4	15.2	17.1
Phenylalanine	72	42.0	31.8	36.6	38.0	47.1
Histidine	25	68.6	61.6	74.3	82.1	78.5
Arginine	63	32.9	42.3	32.4	21.9	12.7
Lysine	123	163.8	190.0	247.1	272.9	

Table 17. Effect of mode and dilution functions
of *V. anguillarum* on trout production.

Inoculation function	24-hr growth		42.5-hr growth	
	C.D.*	LD*/ml	C.D.	LD/ml
Control	0.223	> 300,000	0.410	> 500,000
0-10 hr	0.341	20,000-50,000	0.260	1,000-10,000
0-24 hr	0.341	8,000-10,000	0.250	30,000-50,000
0-65 hr	0.160	20,000-50,000	0.260	20,000-30,000

*C.D. = Optimal density; LD = minimal lethal dose.

Analysis by perfusion and filtration through artificial glass.

A second attempt to substitute fractions of NZ case for the unfractionated NZ case in the standardized medium was made using the acidic, neutral, and basic NZ case fractions prepared by the Dowex and Amberlite resin procedure of Mueller and Miller (1956). Although these workers had found that the three fractions or a combination of the acidic fraction, neutral fraction and acetyl-L-histidine could substitute for unfractionated NZ case in their medium for toxin production, Table 18 shows that this report was not confirmed.

Based on the amino acid analysis of the standardized toxin production medium, a semi-synthetic medium was formulated in which no NZ case was added. The formula for the composition of this medium is given in Table 19. The only ingredient in this table which has a chemically, undefined composition is the beef heart component.

Although this medium supported the growth of unwashed cells of the Mueller strain of C. tetani, no toxin could be detected in the culture filtrate by the mouse assay test. When the medium was supplemented with the ash from 2.25 g NZ case, a value of 10 MLD/ml was obtained by assay of the filtrates of cells grown anaerobically.

When the culture was grown under anaerobic conditions, the potency of the filtrate was less than 1 MLD/ml in mice. A semi-synthetic medium, supplement of acetyl-L-histidine or NZ case ash and acetyl-L-histidine did not improve the yields of toxin obtained using this medium.

A precipitate obtained by treatment of a highly toxic culture filtrate with trichloroacetic acid was digested with trypsin. The digest was highly inhibitory when added to a semi-synthetic medium. Also, a supplement of NZ case ash with a partial acid hydrolyzate of material obtained with trichloroacetic acid resulted in a very low log in growth with no toxin detected.

Table 18. Effect of substitutes for NZ case in the standard medium upon toxin production

Variable	MLD*/ml
NZ case	100,000 and 300,000
Neutral + acidic + basic fractions of NZ case**	10,000 and 50,000
Neutral + acidic fractions of NZ case + acetyl-L-histidine***	1,000 and 10,000

*MLD = minimal lethal dose. Two tests were done.

**Fractions according to procedure of Mueller and Miller (1956).

***21 mg acetyl-L-histidine used per 75 ml medium.

Table 19. Formula lacking IZ case for 100 ml of a
semi-synthetic medium for growth of C. tetani

Ingredient	Concentration
	mg/100 ml
Glucose	2250
NaCl	250
Na ₂ HPO ₄	200
KH ₂ PO ₄	15
MgSO ₄ ·7H ₂ O	15
Lyophilized beef heart broth dialyzate	83.2
Reduced iron	50
L-threonine	29
L-serine	30
L-glutamic acid	29
L-valine	64
L-methionine	32
L-isoleucine (allo free)	32
L-leucine	134
L-tyrosine	88
L-phenylalanine	72
L-lysine.HCl	245
L-histidine.HCl	33
L-arginine.HCl	78
L-cystine	81
Calcium pantothenate	0.100
Uracil	0.250
Thiamin	0.025
Riboflavin	0.025
Pyridoxine	0.025
Biotin	0.00025

[illegible]

The best results were obtained by supplementing the semi-synthetic medium with 18.8 mg tryptophan, 0.20 mg nicotinic acid, 0.060 mg folic acid, 2.5 mg adenine, the ash from 2.25 g NZ base, and the addition of 60 mg threonine or 75 mg serine per 100 ml medium. A value of 30 MID/ml was obtained by assay of the sintered glass filtrate of a 48-hour culture incubated 24 hours at 0-4 C when either excess L-threonine or excess L-serine was used in the medium. Growth was inhibited when an excess (412.8 mg) of L-glutamic acid was used as a supplement to the semi-synthetic medium.

In another attempt to find a good toxin production medium, cells grown in standardized toxin production medium for 12 hours were washed three times in physiological saline and subcultured into medium A (Table 20). As a control to this experiment a medium lacking any nitrogen source was inoculated in the same manner. Medium A supported a yield of toxin (10 MID/ml) while the control medium did not. This medium differed from the standard medium only in the replacement of NZ base by a group of amino acids present in the same proportions as they are in tetanus toxin (Dunn et al., 1948).

It was clear from the results of the preceding experiments that less toxin was produced when various approaches were made to decrease the complexity of the toxin production medium. It seemed that a new approach was needed and it was decided to find or develop a synthetic medium which could be modified until growth with toxin production was obtained.

1. The first part of the paper is devoted to the study of the properties of the function $f(x)$ defined by the equation

$$f(x) = \int_0^x \frac{1}{1+t^2} dt, \quad (1)$$

where x is a real number. It is well known that the function $f(x)$ is an odd function and that

$$f(x) = \arctan x. \quad (2)$$

The function $f(x)$ is also a solution of the differential equation

$$f'(x) = \frac{1}{1+x^2}. \quad (3)$$

It is easy to see that the function $f(x)$ is bounded on the interval $[-\infty, \infty]$ and that

$$\lim_{x \rightarrow \pm\infty} f(x) = \pm \frac{\pi}{2}. \quad (4)$$

The function $f(x)$ is also a solution of the differential equation

$$f''(x) = -\frac{2x}{1+x^2}. \quad (5)$$

It is easy to see that the function $f(x)$ is a solution of the differential equation

$$f'''(x) = \frac{2(1-x^2)}{(1+x^2)^2}. \quad (6)$$

It is easy to see that the function $f(x)$ is a solution of the differential equation

$$f^{(4)}(x) = \frac{8x(1-x^2)}{(1+x^2)^3}. \quad (7)$$

It is easy to see that the function $f(x)$ is a solution of the differential equation

$$f^{(5)}(x) = \frac{8(1-5x^2+x^4)}{(1+x^2)^4}. \quad (8)$$

It is easy to see that the function $f(x)$ is a solution of the differential equation

$$f^{(6)}(x) = \frac{8(1-12x^2+6x^4)}{(1+x^2)^5}. \quad (9)$$

It is easy to see that the function $f(x)$ is a solution of the differential equation

Table 20. Modified standard medium (A) in which NZ case
was replaced by a group of amino acids

Ingredients	Concentration	Medium A	Control Medium
	mg/100 ml		
Glucose	1100.0	+	+
NaCl	250.0	+	+
Na ₂ HPO ₄	200.0	+	+
KH ₂ PO ₄	15.0	+	+
MgSO ₄ ·7H ₂ O	15.0	+	+
FeCl ₃	0.032	+	+
Calcium pantothenate	0.1	+	-
Uracil	0.25	+	-
Thiamin	0.025	+	-
Pyridoxine	0.025	+	-
Biotin	0.00025	+	-
L-valine	54.0	+	-
L-methionine	17.0	+	-
L-threonine	51.0	+	-
L-glutamic acid.HCl	129.0	+	-
L-isoleucine	93.0	+	-
L-leucine	83.0	+	-
L-phenylalanine	50.0	+	-
L-lysine.HCl	151.0	+	-
L-histidine.HCl	15.5	+	-
L-arginine.HCl	40.0	+	-
L-tryptophan	8.2	+	-
L-aspartic acid	15.1	+	-
Glycine	33.3	+	-
L-cystine	25.0	+	-

F. Studies Made of Growth and Toxin Production in a Completely Defined Medium

1. Investigation of the medium formula of Kaufman and Humphries

Kaufman and Humphries (1958) were the only workers who had reported the composition of a synthetic medium for the cultivation of the tetanus organism and they obtained only inconsistent growth in their medium. Furthermore, they did not determine whether their particular strains produced toxin in their medium. Medium prepared according to their formula was tested for its ability to support the growth of the Mueller strain. No growth was obtained and no further work was done with it.

2. Marshall and Kelsey's medium formula modified for study

Marshall and Kelsey (1960) have made studies of various culture media in order to establish a standard medium for general bacteriological usage. In one of their media which appeared to be quite satisfactory for their purposes there were two components, casein digest and yeast autolyzate, the composition of which was determined. Using this analysis and their semi-synthetic medium formula as a basis, a medium was developed which supported the growth of C. tetani. The composition of this synthetic medium, which will be subsequently referred to as S₁ medium, is shown in Table 21.

a. Experiments using unwashed cells

In the first experiment, S₁ medium was autoclaved for 20 minutes at 121 C, cooled, and inoculated with 1.0% of unwashed cells of the MA-8 strain of C. tetani growing in T¹ medium. The medium was then incubated anaerobically at 35 C. The medium did not become turbid until the third day of incubation; at this time only C. tetani cells appeared to be present in a Gram stained smear of the culture. A portion of the culture was

¹T = thioglycollate seed medium.

Table 21. Composition of first successful
synthetic medium (S₁)*

Ingredient	Quantity	Ingredient	Quantity
L-alanine	43.5 mg	CuSO ₄	0.151 mg
L-arginine (free base)	44.0 mg	Na ₂ HPO ₄	0.515 g
L-aspartic acid	72.0 mg	CaCl ₂	0.002 g
L-cystine	5.6 mg	KCl	0.204 g
L-glutamic acid	224.0 mg	Glucose	0.200 g
L-glycine	26.0 mg	β-glycerophosphate, disodium salt	1.0 g
L-histidine	29.7 mg	Thiamin.HCl	0.015 mg
L-isoleucine	66.0 mg	Pyridoxine.HCl	0.015 mg
L-leucine	98.3 mg	Biotin	0.75 ug
L-lysine (free base)	82.7 mg	Calcium pantothenate	0.30 mg
L-methionine	30.9 mg	Riboflavin	0.015 mg
L-phenylalanine	57.2 mg	Nicotinic acid	0.3 mg
L-proline	108.6 mg	Inositol	0.85 mg
L-serine	64.5 mg	B ₁₂	0.009 ug
L-threonine	49.0 mg	Choline chloride	1.0 mg
L-tryptophan	15.5 mg	Folic acid	0.01 mg
L-tyrosine	60.2 mg	Paraaminobenzoic acid	0.003 mg
L-valine	74.3 mg	Solution I**	1.0 ml
MgSO ₄ ·7H ₂ O	20.0 mg	Water, distilled <u>qs</u>	100.0 ml
MnSO ₄ ·4H ₂ O	2.0 mg		
FeSO ₄ ·7H ₂ O	2.0 mg		

*The medium was adjusted to pH 7.0 with diluted sodium hydroxide solution.

**Solution I is composed of 5 ml 85% lactic acid, 8.05 ml distilled water and 2.74 g potassium hydroxide.

harvested then by filtration through sintered glass but the remainder was not harvested until after storage at 4°C for 48 hours. The results of toxicity tests on the two filtrates are shown in Table 22. The results indicated a 10,000-fold increase beyond that carried over in the original inoculum.

From the results of these first experiments it was concluded that an unwashed inoculum of the Et-9 strain of C. tetani could under certain conditions grow in $\frac{1}{2}$ medium and produce toxin.

Experimental work was then characterized by: (i) attempts to grow the organism without resorting to aseptic methods to facilitate metabolic studies at different time intervals; (ii) attempts to sterilize the medium by filtration rather than by heat to avoid misinterpretation of the growth requirements of the organism; (iii) attempts to increase total growth and toxin yield while reducing the lag phase; and (iv) attempts to subculture the organism in relatively solidified synthetic media.

Table 22. Toxicity of culture filtrates
before and after storage at 4 C

Dilutions of the filtrate	Mouse symptoms*			
	Days after mouse injection			
	1	2	3	4
<u>Before storage at 4 C</u>				
100	2+,2+	2+,2+	both dead	
1,000	-, -	-, -	-, -	-, -
10,000	-, -	-, -	-, -	-, -
100,000	-, -	-, -	-, -	-, -
<u>After storage at 4 C</u>				
100	3+,3+	both dead		
500	1+,1+	both dead		
1,000	-, -	2+,2+	both dead	
5,000	-, -	-, -	-, -	1+,1+

*Two mice injected per dilution of the filtrate.

The medium, S₁, was inoculated with unwashed cells,
strain EA-8.












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Experiments were then conducted with the 22-9 strain inoculated into 10 ml of S_1 medium which had been supplemented with sodium thioglycollate and sterilized by sintered glass filtration. After sterilization and before inoculation the supplemented S_1 medium was either heated for 10 minutes at 80 C, heated for 10 minutes at 100 C, or not heated. In unheated medium a visibly turbid growth appeared within 15 hours at 35 C, while in medium preheated at 80 C, 38 hours of incubation were required. No turbidity appeared after 62 hours incubation of the culture in medium that had been exposed to 100 C. From the results of these experiments it was learned that the organism can be cultivated without resorting to anaerobic methods by supplementing the S_1 medium with 50 mg sodium thioglycollate per 100 ml and sterilizing the medium simply by filtration. S_1 medium modified in this manner will hereafter be designated S_2 medium.

Attempts to subculture the organism from S_2 medium to S_2 medium were unsuccessful although the cells were viable for as long as 65 hours after inoculation of the medium as shown by successful transfer of the culture into T medium. It should be noted that the S_2 medium which did not support growth upon subculturing from a previous S_2 medium was prepared on the previous day.

Also, the day old medium did not support growth upon subculturing from a T medium culture unless the S_2 medium was preheated. The heating period here was for 10 minutes at 80 C. It should be noted that there was a 48-hour lag period in the growth of the latter subculture. When a second subculture was attempted from the preheated S_2 medium culture into preheated S_2 medium in other tubes, no growth occurred with aerobic incubation and growth was delayed three days with anaerobic incubation. The results of these studies indicated that the medium required further modification since it supported only delayed subculture growth and this only under anaerobic incubation with preheated medium.

When the S_2 medium was modified by supplementing it with amino acids in the same concentrations as used in the semi-synthetic medium (Table 19), delayed growth (48 hours) occurred with anaerobic conditions. No growth occurred with aerobic conditions. When the S_2 medium was modified by the omission of one or more of the vitamins, inconsistent results were obtained in duplicate tubes of medium or in different experiments. If the medium was brought to a boil by vacuum prior to inoculation, no improvement of growth over the controls could be observed. If the medium was overlaid with mineral oil, growth was far less than in the controls. Inconsistent results were also obtained when 10 ml of S_2 medium were supplemented with 4 mg IT case, 8 mg acetyl-L-histidine or 0.025 mg uvacil. In all the experiments carried out thus far with the S_2 medium, unwashed cells were used as the inoculum. These components of the T medium, or metabolic products of the organism which may have been inhibitory or stimulatory, were introduced into the synthetic medium along with the cells.

b. Experiments using washed cells

In the next series of experiments the inoculum was also prepared by centrifuging and resuspending the cells from an 17 to 24 hour culture in T medium three times in cold buffered saline. Although good growth was evident in less than 15 hours with unwashed cells in S_2 medium, no growth was obtained in this medium with washed cells. Washed cells did grow, however, in T medium after a lag period of more than 15 hours. Such a lag in the growth of washed cells in this richer medium suggested the possibility that during the washing process the cells were injured or these nutrients were removed.

The cells were not significantly injured in the washing procedure since a serial tube dilution of washed cells in T medium indicated the number of cells to be at least 10^6 per 1.0 ml. Unwashed cell inocula contained less than 10^8 per 1.0 ml as determined by the same method at the same time.

An examination of the composition of 0.1 ml of T medium (Table 23) revealed the presence of at least two components which could provide unknown nutrients. These components were yeast extract and Bacto-casitone. When S_2 medium was supplemented with these two components alone or together, the medium did not support growth of a washed inoculum from a 44 hour culture from S_2 medium. As a control 0.1 ml of T medium was added to the S_2 medium and growth was obtained.

Kindler and Meger (1956) reported that carbon dioxide was essential for rapid and regular growth from small inocula of Clostridium parabolulinum type A in partially defined media, and that carbon dioxide could be partially replaced under conditions of strict carbon dioxide depletion by an unidentified factor present in yeast extract. This report provided a basis for one of the variables in the next experiment. In this experiment a solution of sodium bicarbonate was sterilized by Seitz filtration and 0.1 ml of the sterile filtrate was added to 10 ml of S_2 medium so that the amount of sodium bicarbonate added to the medium was 2 mg. In the same experiment 10 mg of autoclaved Darco G 60 were added to 10 ml of S_2 medium as a possible detoxifying agent. A third variable in this experiment was the addition to the S_2 medium of 0.1 ml of a sterile filtrate from the supernatant fluid of cells which were grown for 24 hours in T medium.

Table 23. Composition of 0.1 ml
of thioglycollate seed medium

Ingredient	Amount
Sodium chloride	0.22 mg
Dextrose	0.55 mg
L-cystine	0.05 mg
Agar	0.075 mg
Yeast extract	0.50 mg
Bacto-casitone	1.50 mg
Sodium thioglycollate	0.05 mg
Resazurin	0.0001 mg
Water, distilled, <u>qs</u>	0.10 ml

The experimental results shown in Table 24 indicated that none of these supplements would support the growth of washed cells to the same extent as unwashed cells. Even ten times the number of cells (tubes 13, 14, 15 and 16) gave only sparse growth.

In other experiments with T medium, agar, ash of medium, yeast extract or purified beef heart infusion as supplements to S_2 medium, no growth could be reported with washed cells. Although the unwashed EA-8 strain grew in the S_2 medium, the unwashed Mueller strain did not.

Thus, in the previous series of experiments, the EA-8 strain of C. tetani was grown in T medium and the same number of cells were either washed or inoculated directly into S_2 medium with or without various supplements. Without exception the unwashed cells grew but the washed cells did not grow or grew poorly.

Since the T medium had a small amount of agar which was centrifuged down with the cells during the washing process and which accompanied the cells in the inoculum, it was quite advantageous to learn that the cells could be cultivated in SED¹ which contained no agar. Thus, the cells could be washed free of all contaminants including agar. This new notion was explored in the next experiment.

The results of this experiment (Table 25) indicated that: (i) the best growth was obtained when an unwashed inoculum from T. medium was used to inoculate the unsupplemented synthetic medium; (ii) good growth was also obtained when an unwashed inoculum from SED was used to inoculate the unsupplemented synthetic medium; (iii) if one grew the EA-8 strain in T. medium (SED) first, which the cells could be completely washed, then these cells would grow in the synthetic medium with the proper supplements added; (iv) 0.1 ml

¹SED = stability test broth.

Table 24. Effect of various supplements to S_2 medium upon growth of washed cells (MA-8) from thioglycollate seed medium

Tubes	Inoculum		Supplements		Growth	
	Amt (ml)	Washed	Bicarbonate*	Other	15 hr	63 hr
1,2	0.1	+	-	Thioglycollate filtrate, 0.1 ml	-, -	Good, Good
3,4	0.1	+	+	Thioglycollate filtrate, 0.1 ml	-, -	Good, -
5,6	0.1	+	-	Darco G60, 10 mg	-, -	-, Sparse
7,8	0.1	+	+	Darco G60, 10 mg	-, -	-, Sparse
9,10	0.1	+	+		-, -	-, -
11,12	0.1	+	-		-, -	-, -
13,14	1.0	+	+		Sparse, Sparse	-, -
15,16	1.0	+	-		Sparse, Sparse	-, -
17,18	0.1	+	-		-, -	-, -
19,20	0.1	-	-		Good, Good	Good, Good

*Sodium bicarbonate, 2.0 mg.

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Table 25. Effect of various supplements to S_2 medium upon growth of cells (En-8) from different seed medium

Tubes	Treatment of inoculum	Seed medium*	Supplement	O.D.** at 550 mμ		
				Incubation time		
				16 hr	20.5 hr	39 hr
1,2	Unwashed	STB		0.174	0.194	0.300
3,4	Washed	STB	0.1 ml culture filtrate in STB	0.138	0.135	0.191
5,6	Washed	STB		0.094	0.100	0.110
7,8	Washed	STB	0.1 ml T	0.092	0.100	0.100
9,10	Unwashed	T		0.225	0.325	0.385
11,12	Unwashed	STB	Agar	0.140	0.152	0.270
13,14	Washed	STB	Agar plus 0.1 ml culture filtrate in STB	0.140	0.140	0.235
15,16	Washed	STB	Agar plus STB	0.140	0.155	0.285
17,18	Washed	STB	0.1 ml T medium	0.140	0.174	0.320
19,20	Washed	STB	0.1 ml culture filtrate in T	0.130	0.175	0.305

*STB = sterility test broth; T = thioglycollate seed medium.

**O.D. = average of optical density of duplicate cultures.

amount of F medium is a better supplement for the growth of washed cells in the synthetic medium than a combination of agar and STB; and (v) little or no growth occurred when the synthetic medium was supplemented with 0.1 ml of STB or with no supplement.

The results of this experiment are significant in that for the first time in these studies adequately washed cells were used to obtain growth in a synthetic medium supplemented with only traces of unknown but essential materials.

At about this stage in the work there was difficulty in getting the controls to grow, i.e., unwashed cells from F medium would not grow in the synthetic medium. At first it was theorized that one or more of the vitamin solutions used may have been inactivated but experiments comparing these vitamin solutions to those which had been routinely used did not support this theory. Finally, it was discovered, as can be observed from the results in Table 26, that the sodium thioglycollate used in the synthetic S_0 medium was no longer effective and a fresh supply of the Difco¹ product restored growth in the salt etc. It is interesting to note that growth in the controls always resulted when a solution of ferrous sulphate with the sodium thioglycollate used in the synthetic medium gave a violet color. No growth occurred when this solution was stirred colorless.

The results from this experiment also showed again that the supplemented S_0 medium supported the growth of washed cells from F medium to a greater extent than those grown in STB and that adequately washed cells could grow in the S_0 medium if the medium was supplemented with 0.1 ml F medium or its near equivalent, agar and 0.1 ml STB. The supplement of agar alone had no significant value in supporting growth of the organism.

¹Difco Laboratories, Detroit 1, Mich.

Table 26. Importance of fresh thioglycollate in the preparation of media

Tubes	Treatment of inoculum	Seed medium*	Type sodium thioglycollate	Supplement	O.D.**			
					Incubation time			
					16.5 hr	19.5 hr	24 hr	40 hr
1,2	Unwashed	T	Fresh		0.250	0.285	0.297	0.320
3,4	Unwashed	STB	Fresh		0.162	0.184	0.200	0.198
5,6	Washed	STB	Fresh	0.1 ml T	0.145	0.170	0.207	0.245
7,8	Washed	STB	Fresh	Agar	0.080	0.080	0.093	0.102
9,10	Washed	STB	Fresh	Agar + 0.1 ml STB	0.130	0.145	0.155	0.196
11,12	Unwashed	T	Old		0.030	0.030		
13,14	Unwashed	STB	Old		0.030	0.030		
15,16	Washed	STB	Old	0.1 ml T medium	0.030	0.030		
17,18	Washed	STB	Old	Agar	0.030	0.030		
19,20	Washed	STB	Old	Agar + 0.1 ml STB	0.030	0.030		

*T = thioglycollate seed medium; STB = sterility test broth.

**O.D. = average optical density of duplicate cultures.

PATIENTS		TREATMENT		RESULTS	
No.	Age	Sex	Diagnosis	Duration	Remarks
1	45	M	Chronic	10	Good
2	45	M	Chronic	10	Good
3	45	M	Chronic	10	Good
4	45	M	Chronic	10	Good
5	45	M	Chronic	10	Good
6	45	M	Chronic	10	Good
7	45	M	Chronic	10	Good
8	45	M	Chronic	10	Good
9	45	M	Chronic	10	Good
10	45	M	Chronic	10	Good
11	45	M	Chronic	10	Good
12	45	M	Chronic	10	Good
13	45	M	Chronic	10	Good
14	45	M	Chronic	10	Good
15	45	M	Chronic	10	Good
16	45	M	Chronic	10	Good
17	45	M	Chronic	10	Good
18	45	M	Chronic	10	Good
19	45	M	Chronic	10	Good
20	45	M	Chronic	10	Good
21	45	M	Chronic	10	Good
22	45	M	Chronic	10	Good
23	45	M	Chronic	10	Good
24	45	M	Chronic	10	Good
25	45	M	Chronic	10	Good
26	45	M	Chronic	10	Good
27	45	M	Chronic	10	Good
28	45	M	Chronic	10	Good
29	45	M	Chronic	10	Good
30	45	M	Chronic	10	Good
31	45	M	Chronic	10	Good
32	45	M	Chronic	10	Good
33	45	M	Chronic	10	Good
34	45	M	Chronic	10	Good
35	45	M	Chronic	10	Good
36	45	M	Chronic	10	Good
37	45	M	Chronic	10	Good
38	45	M	Chronic	10	Good
39	45	M	Chronic	10	Good
40	45	M	Chronic	10	Good
41	45	M	Chronic	10	Good
42	45	M	Chronic	10	Good
43	45	M	Chronic	10	Good
44	45	M	Chronic	10	Good
45	45	M	Chronic	10	Good
46	45	M	Chronic	10	Good
47	45	M	Chronic	10	Good
48	45	M	Chronic	10	Good
49	45	M	Chronic	10	Good
50	45	M	Chronic	10	Good
51	45	M	Chronic	10	Good
52	45	M	Chronic	10	Good
53	45	M	Chronic	10	Good
54	45	M	Chronic	10	Good
55	45	M	Chronic	10	Good
56	45	M	Chronic	10	Good
57	45	M	Chronic	10	Good
58	45	M	Chronic	10	Good
59	45	M	Chronic	10	Good
60	45	M	Chronic	10	Good
61	45	M	Chronic	10	Good
62	45	M	Chronic	10	Good
63	45	M	Chronic	10	Good
64	45	M	Chronic	10	Good
65	45	M	Chronic	10	Good
66	45	M	Chronic	10	Good
67	45	M	Chronic	10	Good
68	45	M	Chronic	10	Good
69	45	M	Chronic	10	Good
70	45	M	Chronic	10	Good
71	45	M	Chronic	10	Good
72	45	M	Chronic	10	Good
73	45	M	Chronic	10	Good
74	45	M	Chronic	10	Good
75	45	M	Chronic	10	Good
76	45	M	Chronic	10	Good
77	45	M	Chronic	10	Good
78	45	M	Chronic	10	Good
79	45	M	Chronic	10	Good
80	45	M	Chronic	10	Good
81	45	M	Chronic	10	Good
82	45	M	Chronic	10	Good
83	45	M	Chronic	10	Good
84	45	M	Chronic	10	Good
85	45	M	Chronic	10	Good
86	45	M	Chronic	10	Good
87	45	M	Chronic	10	Good
88	45	M	Chronic	10	Good
89	45	M	Chronic	10	Good
90	45	M	Chronic	10	Good
91	45	M	Chronic	10	Good
92	45	M	Chronic	10	Good
93	45	M	Chronic	10	Good
94	45	M	Chronic	10	Good
95	45	M	Chronic	10	Good
96	45	M	Chronic	10	Good
97	45	M	Chronic	10	Good
98	45	M	Chronic	10	Good
99	45	M	Chronic	10	Good
100	45	M	Chronic	10	Good

The results of an experiment to compare the use of T medium and its component parts as supplements to S_2 medium are recorded in Table 27. Again, unwashed cells required no supplement for growth in the S_2 medium and there was a difference in the growth response depending on the composition of the seed medium. When washed cells were used the best supplement was 0.1 ml of T medium. Since this supplement varied from STB only in its content of agar and resazurin it would be expected that had agar been added to the STB supplement to tubes 11 and 12, growth in these tubes would have been about the same as in tubes 5 and 6. It was shown, however, in tubes 9 and 10 that agar added to STB as a supplement did not increase the amount of growth observed. Perhaps these apparently contradictory results could be explained by the fact that in T medium the components were sterilized by autoclaving; all the components together, whereas the supplements of STB and agar used in tubes 9 and 10 were autoclaved separately and added aseptically to the S_2 medium as supplements.

A comparison of the growth in tubes 13 and 14 to that in tubes 9 and 10 indicated that organic materials in STB stimulated growth since growth was not as good when the ash of STB was substituted for STB. Materials inorganic in nature in STB stimulated growth since if STB ash was omitted little, if any, growth occurred, as seen in tubes 7 and 8. Tubes 7, 8, 15 and 16 indicated that agar alone did not support growth.

To summarize, a supplement of 0.1 ml of T medium per 10 ml of S_2 medium was required for good growth of washed cells in S_2 medium; and T medium probably contained a variety of essential ingredients.

Table 27. The failure of certain components of T medium to maintain growth equal to T medium itself added to S₂ medium

Tubes	Treatment of inoculum	Seed medium*	Supplement	O.D.**	
				Incubation time	
				15 hr	42 hr
1,2	Unwashed	T		0.280	0.310
3,4	Unwashed	STB		0.192	0.220
5,6	Washed	STB	0.1 ml T medium	0.195	0.260
7,8	Washed	STB	Agar	0.105	0.090
9,10	Washed	STB	Agar + 0.1 ml STB	0.135	0.202
11,12	Washed	STB	0.1 ml STB	0.140	0.210
13,14	Washed	STB	Agar + ash of STB	0.126	0.094
15,16	Washed	STB	10-fold agar	0.105	0.091

*T = thioglycollate seed medium; STB = sterility test broth.

**O.D. = average optical density of two cultures.

Since progress in identifying these essential ingredients by supplying individual components of T medium was rather disappointing, it was decided to determine if appropriate supplements added to the S₂ medium in addition to T medium would stimulate greater growth. Experiments were carried out in which variables such as the size of inoculum, various vitamins, NZ case, acetyl-L-histidine, and various purines and pyrimidines were tested but inconsistent results in duplicate cultures were often obtained. When uracil was added as a supplement, however, there was a greater growth response.

The data in Table 28 indicated that the synthetic S₂ medium supplemented with the five purines and pyrimidines would support good growth of the washed cells of the EA-8 strain of C. tetani. If the medium also contained the T medium supplement, better growth was obtained. Growth in the S₂ medium supplemented with uracil was greatly improved by the addition of adenine and good growth was obtained by supplementing the S₂ medium with uracil and hypoxanthine.

Finally, Table 29 shows the composition of 100 ml of a synthetic medium which supported not only the growth of the tetanus organism but also the production of small amounts of tetanus toxin. This formula represents the composition of the medium in its final form as it was developed in this study.

Table 28. Effect of purine and pyrimidine supplements
to S₂ medium* on growth of washed cells (EA-8)

Tube	Supplements**					O.D.*** at 530 mμ					
						Incubation time					
	1	2	3	4	5	0 hr	15.5 hr	20.5 hr	24 hr	39.5 hr	48 hr
1,2	-	-	-	-	-	0.03	0.03	0.03	0.03	0.03	0.03
3,4	+	+	+	+	+	0.040	0.205	0.280	0.302	0.311	0.310
5,6	+	-	+	-	-	0.040	0.200	0.280	0.310	0.320	0.260
7,8	+	-	-	-	-	0.050	0.072	0.080	0.090	0.110	0.120
9,10	+	+	-	-	-	0.040	0.150	0.250	0.250	0.270	0.270

*S₂ medium = synthetic medium containing sodium thioglycollate.

**1 = Uracil; 2 = Adenine; 3 = Hypoxanthine; 4 = Cytosine; 5 = Thymine.

***O.D. = average optical density of duplicate cultures.

Table 29. Final composition of a synthetic medium
adequate for growth with some toxin production

Ingredient	Quantity	Ingredient	Quantity
L-alanine	43.5 mg	KCl	0.204 g
L-arginine	44.0 mg	Glucose	0.200 g
L-aspartic acid	72.0 mg	Thiamin	0.015 mg
L-cystine	5.6 mg	Pyridoxine.HCl	0.015 mg
L-glutamic acid	224.0 mg	Biotin	0.75 ug
L-glycine	26.0 mg	Calcium pantothenate	0.30 mg
L-histidine (free base)	29.7 mg	B ₁₂	0.009 ug
L-isoleucine	66.0 mg	Choline chloride	1.0 mg
L-leucine	98.3 mg	Folic acid	0.01 mg
L-lysine (free base)	82.7 mg	Paraaminobenzoic acid	0.003 mg
L-methionine	30.9 mg	Riboflavin	0.015 mg
L-phenylalanine	57.2 mg	Nicotinic acid	0.3 mg
L-proline	108.6 mg	Inositol	0.85 mg
L-serine	64.5 mg	Uracil	0.75 mg
L-threonine	49.0 mg	Adenine	0.250 mg
L-tryptophan	15.5 mg	Hypoxanthine	0.250 mg
L-tyrosine	60.2 mg	Cytosine	0.250 mg
L-valine	74.3 mg	Thymine	0.250 mg
β -glycerophosphate, disodium salt	1.0 g	Solution I*	0.5 ml
Sodium thioglycollate	0.050 g	Solution II**	1.0 ml
H ₂ HPO ₄	0.151 g	Solution III***	1.0 ml
CaCl ₂	0.002 g	Water, distilled <u>qs</u>	100.0 ml

*Solution I is composed of 0.4 g $\text{H}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g $\text{Na}_2\text{SO}_4 \cdot 4\text{H}_2\text{O}$, 0.04 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 ml distilled H_2O .

**Solution II is composed of 0.0151 g CuSO_4 and 100 ml distilled H_2O .

***Solution III is composed of 5 ml 85% lactic acid, 6.05 ml distilled H_2O , and 2.74 g of KOH.

The medium is prepared according to the following steps:

(i) All liters in the first column of Table 29 except cystine and tyrosine are weighed into a 200 ml beaker.

(ii) Cystine and tyrosine are dissolved in 1.25 N HCl and added to the beaker.

(iii) A 100 ml amount of distilled water is added to the beaker and the solution is warmed to 35 C and stirred.

(iv) A 0.5 ml amount of Solution I is added to the beaker. Solution I is composed of 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.04 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 ml distilled water.

(v) A 1.0 ml amount of Solution II is added to the beaker. Solution II is composed of 0.0151 g CuSO_4 and 100 ml distilled water.

(vi) A 1.0 ml amount of Solution III is added to the beaker. Solution III is composed of 5 ml 85% lactic acid, 8.05 ml distilled water, and 2.74 g of KOH.

(vii) A 0.01 ml amount of a stock solution of thiamin is added to the beaker. The stock solution contains 75 mg of thiamin and 50 ml distilled water.

(viii) A 0.01 ml amount of a stock solution of pyridoxine.HCl is added to the beaker. The stock solution contains 75 mg of pyridoxine.HCl and 50 ml distilled water.

(ix) A 0.05 ml amount of a stock solution of biotin is added to the beaker. The stock solution contains 7.5 mg biotin and 500 ml 95% ethanol.

(x) A 0.05 ml amount of a stock solution of calcium pantothenate is added to the beaker. The stock solution contains 300 mg calcium pantothenate and 50 ml of distilled water.

2. 凡在中华人民共和国领域内从事生产经营活动的法人和其他组织，均应当依照本法的规定，向税务机关申报纳税。（）

3. 纳税人应当依照本法的规定，如实申报纳税，不得隐瞒应纳税额，不得虚报减免税。（）

4. 纳税人应当按照国家有关规定，依法设置账簿，并妥善保管。（）

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(xi) A 0.05 ml amount of a stock solution of B₁₂ is added to the beaker. To prepare this solution, 0.7 ml of a solution of 15 ug of B₁₂ per liter of 95% EtOH is added to 104.3 ml of 25% ethanol.

(xii) A 0.1 ml amount of a stock solution of choline chloride is added to the beaker. The stock solution contains 25 mg of choline chloride and 2.5 ml distilled water.

(xiii) A 0.1 ml amount of a stock solution of folic acid is added to the beaker. The stock solution contains 1 ml of a solution of 9 mg folic acid per 10 ml distilled water and 9 ml of distilled water.

(xiv) A 0.1 ml amount of a 1:10 dilution of a solution of 3 mg p-aminobenzoic acid per 10 ml distilled water is added to the beaker.

(xv) A 0.01 ml amount of a solution of 3 mg of riboflavin per 2.0 ml of water is added to the beaker.

(xvi) A 0.1 ml amount of a solution of 3 mg of nicotinic acid per 1.0 ml distilled water is added to the beaker.

(xvii) A 0.1 ml amount of a solution of 8.5 mg inositol per 1.0 ml distilled water is added to the beaker.

(xviii) A 1.0 ml amount of a solution of 7.5 mg uracil per 10 ml of distilled water is added to the beaker.

(xix) A 1.0 ml amount of a solution of 2.5 mg of adenine per 10 ml distilled water is added to the beaker.

(xx) A 1.0 ml amount of a solution of 2.5 mg hypoxanthine per 10 ml of distilled water is added to the beaker.

(xxi) A 1.0 ml amount of a solution of 2.5 mg cytosine per 10 ml distilled water is added to the beaker.

(xxii) A 1.0 ml amount of a solution of 2.5 mg thymine per 10 ml distilled water is added to the beaker.

(xiii) The solution in the beaker is now adjusted to pH 7.0 with 2 N sodium hydroxide. The O. R. of the sodium is 0.0 volts.

(xiv) The contents of the beaker are filtered through a clean and sterile sintered glass filter.

This medium when prepared in 100 ml or larger volumes supported the growth of washed cells of either the EA-8 or Mueller strains of the tetanus organism. Both strains produced as much as 10 MID/ml of toxin in the medium after an incubation period of 23 hours and both cultures contained 10^7 to 10^8 cells per ml at this time as measured by the serial tube dilution method. The highest toxin titer which was obtained in this medium was obtained using the EA-8 strain of the organism grown in 75 ml of synthetic medium for 24 hours at 35 C followed by another 24 hours at 0-4 C prior to the sterile filtration of the culture using a sintered glass filter. Fifty ml of the filtrate were harvested and the mouse MID value was 100/ml. Toxic symptoms occurred in the mice at a dilution of 1 to 500.

Also, the strain was successfully subcultured in this synthetic medium on two successive days using 0.1 ml of inoculum per 10 ml of medium. Two-dimensional paper chromatography gave the same spectra of ninhydrin positive materials after growth in the synthetic medium as it did before inoculation.

V. DISCUSSION

In the production of tetanus toxin certain factors influenced the yields obtained in a good growth medium. First, it was necessary to keep easily oxidizable ingredients, such as sodium thioglycollate, sealed and protected from inactivating agents. Second, it was necessary to prepare sufficiently large batches of media to avoid significant adsorption of trace materials during sterile filtration.

A third factor which affected the yields of toxin was the growth phase of the culture at the time of harvest as determined microscopically. The extent of autolysis correlated very well with the quantity of trichloroacetic acid precipitable material and MLD values which were obtained on the culture filtrate. Lysis could be induced by chilling, freezing and thawing, or treatment with an optimum amount of penicillin G. Lysis was induced in order to determine the total amount of toxin which had been produced at any time after inoculation of the medium with the organism. Penicillin was used as the lysing agent since it has been shown to be most effective in its action against susceptible bacteria when the cells were rapidly metabolizing or in the log phase of growth. It was found in this study that toxin was produced at the greatest rate while the cells were in the log phase and that the addition of larger amounts of penicillin resulted in lower toxin yields. It is interesting to note the therapeutic implications involved here since penicillin is prescribed along with tetanus antitoxin for the treatment of tetanus patients. Doctors should be sure that patients have circulating antibodies sufficient to neutralize the toxin liberated by penicillin treatment.

A fourth factor which determined the yields concerned the method of filtering the toxin. The yields of toxin were considerably lower and less uniform when the filtrates from stainless steel filters (fitted with $1\frac{1}{2}$ inch diameter, 33 Seitz asbestos filter pads) were compared to those from sintered glass filters of the same size. The explanation for this could be in the asbestos-type filter pads containing heavy concentrations of calcium, magnesium, and iron which are not easily recovered.

A fifth factor of importance involved the nature of the toxin and the assay of it. The toxin is capable of undergoing detoxification spontaneously in a manner not completely understood. The fact that this reaction can occur affects the choice of potency measurement and interpretation of the results. The IID value is a measure of only the toxicity of the sample. The LD value is the measure of both the toxin and detoxified portions of the sample since antitoxin combines with both toxin and toxoid. One LD unit is equivalent to about 10,000 IID (Muller & Muller, 1954). The toxin test is considered the best measure of the toxicity of a sample of greater than 1,000 IID/ml since 0.2 unit of antitoxin will inactivate 1,000 IID of toxin, an equivalent amount of toxoid, or the same amount of a mixture of toxin and toxoid. The culture filtrates obtained from the synthetic media were not assayed for LD or LD₅₀ values since the IID values were below the range of these values, and the trichloroacetic acid precipitable material was barely visible.

The four-portioned line method as well as pH, ionic strength, protein concentration and temperature affect the activity of tubercle toxin.

Two approaches were taken in this study to prepare a successful formula for a synthetic medium which would support production of tetanus toxin. These approaches consisted of attempts either to simplify a good complex medium or to devise a synthetic medium supporting both growth of the organism and toxin production. In spite of the reported requirements for peptides in a complex medium and the unavailability of an adequate synthetic medium it was decided first to undertake the difficult task of simplifying the formula for the complex medium of Mueller and Miller (1956).

The complexity of the medium was indicated by the high per cent of peptide nitrogen (Table 12) which was present in HZ case and by the beef heart requirement (Table 1) in Mueller and Miller's formula. Since the organism could grow and produce toxin in a cellophane bag (Figure 1) which would exclude materials of large molecular weight from the cells, it seemed logical to simplify the medium by fractional dialysis of the two ingredients and to identify chemically their composition. Apparently the method did not resolve the substances in the medium which were stimulatory from those which were inhibitory. Nevertheless, this procedure did produce a standardized medium which contained no trichloroacetic acid precipitable components and which supported good growth with toxin production.

The amino acid analyses which were made on the standardized medium prior to its inoculation with the organism provided a basis for a semi-synthetic medium. The changes in the concentrations of these amino acids at different incubation times could not be evaluated since all ninhydrin positive materials were not identified.

During studies carried out towards reaching the goal by appropriate modification of a synthetic medium which supported growth, precautions were taken to prevent introduction of heat-promoted inhibitory substances during autoclaving. Fox (1956) has shown that amino acids can polymerize under heat and pressure. Lackford et al. (1957) have pointed out that destructive effects occur in media which contain glucose and cystine when these media are sterilized by autoclaving. On the other hand Miller (1955b) has reported that glucose cannot be sterilized separately and added to NZ case production medium without a loss in toxin production.

These problems were avoided by warming the medium to only 35 C for dissolving all the ingredients. Cystine and tyrosine were dissolved in 1.25 N HCl and added to the medium before the medium was sterilized by filtration through a sintered glass filter. The results obtained with the organism in media made up in 20 ml amounts were frequently inconsistent. The reason for this is not known; however, it can be avoided by increasing the quantity of the medium to be filtered.

Since growth with toxin production in an unheated medium which contained no peptides was obtained with cells washed three times with cold buffered saline, it can be concluded that peptides are not essential for toxin production. This conclusion is not in disagreement with the suggestion that peptides stimulate toxin production in a complex medium. In a complex medium there may be substances which inhibit amino acid assimilation or the cell may be more readily permeable to amino acids when in peptide linkage. Another explanation commonly given for the superiority of peptides over amino acids as nutrients is that amino acids may be degraded more rapidly than peptides with the result that amino acid deficiencies are established. The reason histidine peptides were found by Mueller and

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Miller (1956) to be essential for toxin production by Clostridium tetani in a complex medium was not determined. The fact that these workers found the same peptides were not essential for growth is a striking feature in the study of the metabolism of this organism. In the large scale production of tetanus toxin a more rapid rate of growth without a corresponding increase in the toxin yields occurs when a peptic digest medium (Michigan Department of Health, 1958) is used in place of the medium described by Mueller and Miller (1954). It is also interesting that the toxin culture filtrate obtained using the peptic digest medium is superior to that obtained using the Mueller and Miller medium formula for the production of tetanus antitoxin in the horse.

The effect of reduced iron on toxin production in the two media is still unexplained. The optimum concentration in the peptic digest medium appears to be twice the optimum concentration in the medium prepared by Mueller and Miller's formula (1954).

The synthetic medium formulated in this study provides a basis for future investigations on the nutrient requirements of the organism with respect to the production of tetanus toxin. It opens the door to studies which might lead to the establishment of metabolic pathways for the utilization of glucose and for studies concerning protein synthesis. In this connection it should be pointed out that the metabolism of D-glycerol phosphate has not been studied very extensively. In its present form, however, the synthetic medium has an excessive number of components to prepare and is too expensive for any practical importance. The chief advantage of the use of a synthetic medium for toxin production is the ease of making physiological studies and the purification of metabolic products.

VI. SUMMARY

A complex medium which supported a good yield of tetanus toxin by the Mueller strain of Clostridium tetani was standardized and simplified by replacing beef heart infusion with a beef heart dialyzate. During the entire growth period changes in the concentrations of free amino acids were determined. Threonine, serine, and glutamic acid were consumed completely during the first day when the rate of toxin production was maximal. Penicillin acting as a lysing agent gave increased yields of toxin.

A further simplification of the Mueller medium was achieved by substituting a group of amino acids for the IS case which successfully supported a small amount of toxin production from a small unwashed inoculum.

Finally, a chemically defined medium was prepared which contained no peptides and supported reasonable growth with measurable toxin production from a washed inoculum of two commercial strains.

BIBLIOGRAPHY

- Block, R.J., E. L. Durrum, and G. Zweig. 1955. Amino acids, amines, and proteins. Section 1: Amino acids and peptides, p. 75-113. In A manual of paper chromatography and paper electrophoresis. Academic Press, Inc., New York, N.Y.
- Clifton, C.E. 1942. The utilization of amino acids and related compounds by Clostridium tetani. J. Bacteriol. 44:179-183.
- Craig, L. C., J. D. Gregory, and W. Hausman. 1950. Versatile laboratory concentration device. Anal. Chem. 22:1462.
- Craig, L. C., and T. P. King. 1955. Some dialysis experiments with polypeptides. J. Am. Chem. Soc. 77:6620-6624.
- Descombey, P. 1924. L'anatoxine tetanique. C. R. Soc. Biol. Paris. 91:239-241.
- Dunn, M. S., M. N. Camien, and L. Pillemer. 1949. The amino acid composition of tetanal toxin. Arch. Biochem. 22:374-376.
- Eaton, L. M., E. H. Lambert, M. A. Christensen, D. L. Thurber, E. D. Henderson, and H. H. Young. 1957. Symposium on tetanus. Proc. Staff Meetings of Mayo Clinic. 32:141-167.
- Faber, K. 1890. Die pathogenese des tetanus. Berl. Klin. Wschr. 27:717-720.
- Fedinec, A. A., and H. A. Matzke. 1958. The role of tissue spaces and nerve fibers in the spread of tetanus toxin in the rat. Univ. Kansas Sci. Bull. 38:1439-1498.
- Feeney, R. E., J. H. Mueller, and P. A. Miller. 1943a. Growth requirements of Clostridium tetani. II. Factors exhausted by growth of the organism. J. Bacteriol. 46:559-562.
- Feeney, R. E., J. H. Mueller, and P. A. Miller. 1943b. Growth requirements of Clostridium tetani. III. A "synthetic" medium. J. Bacteriol. 46:563-571.
- Fildes, P., W. Bullock, R. A. O'Brien, and A. T. Glenn. 1929. Bacillus Tetani, p. 298-372. In A system of bacteriology in relation to medicine. V. 3. His Majesty's Stationery Office, London.
- Fisek, H. H., J. H. Mueller, and P. A. Miller. 1954. Muscle extractives in the production of tetanus toxin. J. Bacteriol. 67:329-334.
- Fox, S. V. 1956. Evolution of protein molecules and thermal synthesis of biochemical substances. Am. Scientist. 44:347-359.

- Hamilton, P. H. 1950. Ion exchange chromatography of amino acids. Effect of resin particle size on column performance. Anal. Chem. 30:914-918.
- Kaufman, L., and J. J. Humphries. 1958. Studies of the nutritional requirements of Clostridium tetani. I. A chemically defined medium. Appl. Microbiol. 6:311-315.
- Kindler, S. H., and J. Luger. 1956. Nutritional studies with the Clostridium botulinum group. J. Gen. Microbiol. 15:386-393.
- Kitasato, S. 1889. Ueber den tetanus bacillus. D. Hyg. Infektkr. 7:225-234.
- Koch, W., and D. Kaplan. 1953. A simple method for obtaining highly potent tetanus toxin. J. Immunol. 70:1-5.
- Leakford, C. E., J. A. Ravel, and H. H. Ramsey. 1957. The effect of glucose and heat sterilization on bacterial assimilation of cystine. Appl. Microbiol. 5:65-69.
- Lempier, J. F. 1956. Investigation of the tetanus toxin from two different strains of Clostridium tetani. J. Immunol. 76:393-396.
- Lerner, E. H., and J. H. Mueller. 1949. The role of glutamine in the glucose metabolism of Clostridium tetani. J. Biol. Chem. 181:42-45.
- Lerner, E. H., and M. J. Pickett. 1945. The fermentation of glucose by Clostridium tetani. Arch. Biochem. 8:183-196.
- Marshall, J. H., and J. J. Hilsey. 1960. A standard culture medium for general bacteriology. J. Hygiene. 58:367-372.
- Martinez, R. J., and S. J. Wittenberg. 1959. Glucose dissimilation by Clostridium tetani. J. Bacteriol. 77:156-163.
- Michigan Department of Health, Division of Laboratories. 1958. Tetanus toxin and toxoid production outline. Lansing, Michigan.
- Miller, P. A. 1955a. Histidine peptides essential for the formation of tetanus toxin. Bacteriol. Proc. 1955:126.
- Miller, P. A. 1955b. Personal Communication. Dept. Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts.
- Miller, P. A., C. E. Gray, and H. D. Ester. 1956. A system for studying tetanus toxin formation. Bacteriol. Proc. 1956:116.
- de Chassy, H., and J. Jodogne. 1958. Mise en application des theories sur l'extraction de la toxine tetanique endocellulaire. I. Action de la penicilline. Ann. Inst. Pasteur. 14:386-399.
- Mooney, P. J., and J. H. Taylor. 1932. The preparation of stabilized toxoid toxin. Am. J. Pub. Health. 22:43-49.

- Moore, S., and W. H. Stein. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* 176:367-388.
- Moore, S., and W. H. Stein. 1951. Chromatography of amino acids on sulfonated polystyrene resins. *J. Biol. Chem.* 192:663-681.
- Moore, S., and W. H. Stein. 1954. Procedures for the chromatographic determination of amino acids on four per cent cross-linked sulfonated polystyrene resins. *J. Biol. Chem.* 211:893-906.
- Moore, S., D. H. Spackman, and W. H. Stein. 1958. Chromatography of amino acids on sulfonated polystyrene resins. *Anal. Chem.* 30:1185-1190.
- Mueller, J. H., and P. A. Miller. 1940. Tetanus toxin production on a simplified medium. *Proc. Soc. Exper. Biol. and Med.* 43:389-390.
- Mueller, J. H., and P. A. Miller. 1942. Growth requirements of *Clostridium tetani*. *J. Bacteriol.* 43:763-772.
- Mueller, J. H., and P. A. Miller. 1943. Large-scale production of tetanal toxin on a peptone-free medium. *J. Immunol.* 47:15-22.
- Mueller, J. H., and P. A. Miller. 1945. Production of tetanal toxin. *J. Immunol.* 50:377-384.
- Mueller, J. H., and P. A. Miller. 1947. Factors influencing the production of tetanal toxin. *J. Immunol.* 56:143-147.
- Mueller, J. H., and P. A. Miller. 1954. Variable factors influencing the production of tetanus toxin. *J. Bacteriol.* 67:271-277.
- Mueller, J. H., and P. A. Miller. 1955. Separation from tryptic digests of groups of amino acid-labile components essential in tetanus toxin formation. *J. Bacteriol.* 62:634-642.
- Mueller, J. H., and P. A. Miller. 1956. Essential role of histidine peptides in tetanus toxin production. *J. Biol. Chem.* 223:197-194.
- Mueller, J. H., H. B. Taboanbaal, J. J. Jezukewicz, and P. A. Miller. 1943. Production of tetanus toxin on peptone-free media. *J. Clin. Investigation.* 22:315-316.
- National Institutes of Health. 1952. Minimum requirements: Tetanus toxoid. Bethesda Md, Maryland.
- Oakley, C. L. 1954. Bacterial toxins. *Ann. Rev. of Microbiol.* 8:411-426.
- Oakley, C. L. 1957. Bacterial toxins and classification. *Gen. Microbiol.* 10:344-347.
- Pillay, R., A. Whittier, and D. H. Crosskey. 1946. The isolation and crystallization of tetanal toxin. *Canad. J.* 10:615-616.

- Pillay, L., R. Whittler, J. Burrell, and D. B. Crossman. 1946. The immunochemistry of toxins and toxoids. VI. The crystallization and characterization of tetanal toxin. *J. Exper. Med.* 68:205-221.
- Pillay, L., and K. J. Robbins. 1948. Chemistry of toxins. *Ann. Rev. of Microbiol.* 3:265-288.
- Prevot, L. P., and H. J. Boersma. 1941. On the subject of the fermentation of glucose by Clostridium tetani. *Antonie van Leeuwenhoek, J. Microbiol. and Serol.* 7:232.
- Rinker, J. A. 1957. Properties of bacterial flagella. Ph.D. thesis. Purdue University, Lafayette, Indiana.
- Rosenthal, E. 1937. "Oxoid-sulphuric acid" method for anaerobic cultures. *J. Bacteriol.* 34:317-320.
- Stone, J. E. 1953. A modified Mueller medium without native protein for tetanus toxin production. *Appl. Microbiol.* 1:166-167.
- Tamm, H., and R. B. F. A. Pordman. 1941. On the fermentation of glucose by Clostridium tetani. *Antonie van Leeuwenhoek, J. Microbiol. and Serol.* 7:242.
- The son, R. O. 1954. A semi-continuous method for the large-scale production of tetanus toxin. *Nature.* 180:1126-1127.
- Thompson, H., and H. Heyland. 1958. Tetanus toxin. *Ann. Ent. Inst.* 51:773.
- van Heyningen, W. H. 1950. Bacterial toxins. Blackwell Scientific Publications, Oxford, 2 Gluck. 366 p.
- van Heyningen, W. H. 1956. The reactions of toxins with antibodies. *Dissem. Soc. of p.* No. 10:37-46.
- Venglar, V. C., and W. G. Remy. 1942. Cellular toxins or the infectious factors in the causation of disease. Ben Brothman and Company, P. and H. K. New edition, 51-56.
- Von Tschiling, H., and C. Kitzinger. 1939. Ueber das zuckerfermentierende Verhalten - Immunität und das tetanus immunisat bei tieren. *Deutsch. Ab. Schmeider.* 16:1113-1114.
- Wright, G. P. 1955a. Tetanus and tetanus toxins. *Typ. Soc. Gen. Microbiol.*, No. V, *Advances of Microbial Pathogenicity*:78:192.
- Wright, G. P. 1955b. The neurotoxins of Clostridium botulinum and Clostridium tetani. *Pharmacol. Revs.* 7:413-465.

1. The first part of the paper discusses the importance of the study of the history of the United States. It is argued that a knowledge of the past is essential for a full understanding of the present and for the development of a sound policy for the future. The author points out that the study of history is not only a means of learning about the past, but also a way of developing the ability to think critically and to make sound judgments.

2. The second part of the paper discusses the role of the government in the development of the United States. It is argued that the government has played a crucial role in the development of the country, and that it is essential for the government to continue to play this role in the future. The author points out that the government has been responsible for the establishment of the Constitution, the development of the federal system, and the creation of the various departments and agencies of the government.

3. The third part of the paper discusses the role of the individual in the development of the United States. It is argued that the individual has played a crucial role in the development of the country, and that it is essential for the individual to continue to play this role in the future. The author points out that the individual has been responsible for the establishment of the various states, the development of the various industries, and the creation of the various social and economic institutions of the country.

4. The fourth part of the paper discusses the role of the future in the development of the United States. It is argued that the future is a time of great opportunity, and that it is essential for the United States to continue to develop and to grow. The author points out that the future is a time when the United States will be able to realize its full potential, and when it will be able to play a leading role in the world.

5. The fifth part of the paper discusses the role of the United States in the world. It is argued that the United States has a special responsibility in the world, and that it is essential for the United States to continue to play this role in the future. The author points out that the United States has been responsible for the establishment of the United Nations, the development of the various international organizations, and the creation of the various international treaties and agreements.

6. The sixth part of the paper discusses the role of the United States in the development of the world. It is argued that the United States has a special responsibility in the development of the world, and that it is essential for the United States to continue to play this role in the future. The author points out that the United States has been responsible for the establishment of the various international organizations, the development of the various international treaties and agreements, and the creation of the various international institutions of the world.

7. The seventh part of the paper discusses the role of the United States in the development of the future. It is argued that the United States has a special responsibility in the development of the future, and that it is essential for the United States to continue to play this role in the future. The author points out that the United States has been responsible for the establishment of the various international organizations, the development of the various international treaties and agreements, and the creation of the various international institutions of the future.

8. The eighth part of the paper discusses the role of the United States in the development of the world and the future. It is argued that the United States has a special responsibility in the development of the world and the future, and that it is essential for the United States to continue to play this role in the future. The author points out that the United States has been responsible for the establishment of the various international organizations, the development of the various international treaties and agreements, and the creation of the various international institutions of the world and the future.

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