THE DEVELOPMENT OF CLOSTRIDIUM BOTULINUM TYPES 62 A AND B IN STERILE SKIMMILK

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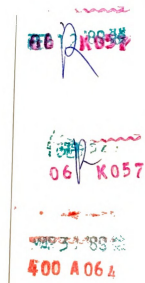

ABSTRACT

THE DEVELOPMENT OF CLOSTRIDIUM BOTULINUM TYPES 62 A AND B IN STERILE SKIMMILK

by André R. Brillaud

Culture development of Clostridium botulinum Types 62 A and B in sterile skimmilk, at initial levels of 10,000, 1,000, 100 and 10 per ml, and stored at 18, 13, 10 and 7 C, was studied. Growth of Type 62 A occurred at all levels of inoculation when sample was incubated at 18 and 13 C. Toxin was produced and visual and olfactory changes also occurred with culture development in the skimmilk medium. At 10 and 7 C no growth, toxin production, visual or olfactory changes were evident. Toxin appeared in all cases of culture development as the population attained a level of greater than 1.0×10^5 per ml. Immediately prior to the appearance of toxin in the medium an amino odor was always detected. Lethal levels of toxin were present before visual and other olfactory changes became evident.

Clostridium botulinum Type B grew in sterile skimmilk when incubated at 18 C at initial inocula levels of 1,000, and 100 per ml. The data obtained on the growth of Type B compared well with the data obtained with Type 62 A.

The inhibitory effect of the following fatty acids: butyric, oleic, caproic, caprylic, capric, lauric, myristic, palmitic on the culture development of Type 62 A was tested. The individual acids were tested at 0.01% in skimmilk and 0.001% in a 4% Trypticase medium. Caprylic, capric, myristic and stearic acids were found to have an

effect in prolonging the lag phase of culture development. Mixtures of all the acids were more effective in prolonging the lag phase.

Monolayer cell tissue cultures were studied to learn if they were sensitive to the cytotoxic action of botulinus toxin. Mouse C_3H, Osgood J-111, Mouse L Cell, Detroit-6-Parent, Detroit-6-Yale Clone and Embryo Monkey Kidney cell cultures were used. Purified Type A toxin and toxin produced in skimmilk were tested. All cultures were found to have varying sensitivities to the toxin with the exception of Mouse L cell which was refractive to the toxin. Heat inactivated toxin and specific antitoxin were used as controls.

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The successful completion of this work was made possible by the help and confidence given to me by my wife, Joan, to whom this work is dedicated.

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INTRODUCTION

The main objective in pasteurizing milk at 62 C for 30 min or at 72 C for 16 sec is to destroy the nonsporeforming disease-producing bacteria. Nonpathogenic thermoduric bacteria belonging to the genus Micrococcus and heat-resistant sporeformers belonging to the genus Clostridium or Bacillus will survive these pasteurization procedures if they are present in raw milk (Martin et al., 1961).

The presence of clostridial spores in pasteurized milk as a result of pre- or post-pasteurization contamination, however, has never presented a public health problem as no deaths from botulinus poisoning have ever been reported from milk. The relative short storage period encountered in the dairy industry today, as well as the low storage temperature undoubtedly exert some controlling influence. Studies involving prolonged holding periods at low temperatures are particularly important in view of the new advances in pasteurization which provide for a considerable increase in the shelf-life of pasteurized milk. The advent of ultra-high temperature (UHT) pasteurization which utilizes temperatures from 88 to 121 C with "no intended holding period" has increased the storage life of milk but the milk is not sterile (Tobias et al., 1955). This study was undertaken to determine the development of a culture of C. botulinum in sterile skimmilk on prolonged storage at low temperatures.

Particular attention was directed to the effect of fatty acids on culture development since it has been reported (Foster and Wynne, 1948) that oleic, linoleic, and linolenic acids inhibited the germination of spores of C. botulinum but did not affect the growth of vegetative cells.

An investigation was also undertaken to study the effect of botulinus toxin on tissue cell cultures. Tissue culture systems were selected as some information is available on detection of toxic material by this procedure (Rightsel et al., 1958; Rope et al., 1957).

REVIEW OF THE LITERATURE

The genus Clostridium comprises a large group of organisms, some of which can be divided into two subgroups according to their physiological manisfestations. The first is the food poisoning group; the most well-known member being Clostridium botulinum. The second group is comprised of bacteria which cause histotoxic infections in man and other animals. The best known member of the latter group is Clostridium perfringens, also known as Clostridium welchii. The food poisoning group is significant since they cause toxicity without in vivo growth and multiplication, while the latter, the gas gangrene group, produces infections in vivo.

The members of the genus Clostridium are sporeforming, anaerobic, rod-shaped bacteria which, under appropriate conditions produce neurotoxins that are among the most potent poisons known. Clostridium botulinum produces a neurotoxin which is released into the growth medium after the major increase in the bacterial population has occurred (Lamanna, 1959). The organism is generally unable to grow and produce toxin within the bodies of homeothermic animals. They are generally not linked to an infectious process. Botulism is, therefore, a matter of food poisoning. Different strains of C. botulinum produce antigenically distinguishable neurotoxins and the injection of graduated doses will stimulate the production of antitoxins in animals. The several toxins of C. botulinum are distinguished serologically (Oakley, 1954) and typed according to their antigenicity as A, B, C, D and E. Types A, B, and E are most frequently detected in food poisonings in man. Bronfenbrenner et al. (1921) have reported strain differentiation by immunological methods and Gunnison and Scheonholz

(1927a, b) have shown serological classification by complement fixation and precipitin reactions. The identification of botulinus toxin in canned foodstuffs by serological methods has been reported by Keiser (1923).

A number of investigators have studied the development of spores of C. botulinum in various foods. Halvorson (1949) studied botulism in canned bread, Segalove and Dack (1951) studied the growth of the culture in dehydrated meat, Wagenaar and Dack (1954, 1955, and 1958) investigated the development of the culture in canned bread, cheese, and cheese spread, and Grecz et al. (1959a, b) studied the development of C. botulinum in cheese. No data are available on the development of the culture in milk. Other investigators have studied the factors influencing the inhibition of growth, toxin production, and spore germination of C. botulinum by various substances and/or compounds. Bonventre and Kempe (1959) and Tanner et al. (1939) studied the effect of pH and temperature on the culture development of spores of C. botulinum in a medium consisting of casein and yeast extract. They found that the organism would not grow when the pH was 4.5 or lower and the temperature was 5 C or lower. At 10 C Tanner et al. (1939) found that toxin production was negligible. The effect of canning of corn and mushrooms on the culture development of C. botulinum was studied by Denny et al. (1961). Grecz et al. (1959c, 1961) reported that the growth limiting total fatty acid concentration for C. botulinum in aged cheese was approximately 2%. They also reported the production of antimicrobial agents, presumably fatty acids, in surface ripened cheese. Humfield (1947) reported on the inhibitory action of fatty acid-like constituents of wheat on germination of Clostridium spores. Germination of spores of C. botulinum was shown to be inhibited by

oxidative rancidity in unsaturated fatty acids (Roth and Halvorson, 1952). Tarr (1953) and Townsend et al. (1954) studied the effect of amines and acidity on the germination and growth of C. botulinum in fish muscle and Trypticase medium. Foster and Wynne (1948) reported on the inhibition of germination of spores of C. botulinum by unsaturated C_{18} fatty acids.

Little or no information is available on culture development and toxin production of C. botulinum in milk stored for long periods of time at low temperatures. Similarly, no information is available on the visual and olfactory changes which occur when this organism grows in milk. Some research has been conducted, however, on the inhibition of germination of endospores of C. botulinum, C. pasteurianum, Bacillus subtilis, and B. stearothermophilus by oxidized unsaturated fatty acids (oleic, linoleic, and linolenic) at a concentration of 0.1% (Roth and Halvorson, 1952). Krukovsky (1961) stated that there is an increase of lipolytic rancidity and acidity in unpasteurized milk which had been aged; the concentration of fatty acid is directly associated to the time of storage. Foster and Wynne (1948) reported the inhibition of germination of spores of C. botulinum in synthetic growth medium using 0.01% oleic acid and higher concentrations of linoleic and linolenic acids. In bovine milk production environmental temperature and humidity affected the fatty acid composition of the product (Richardson et al., 1961), hence the fatty acid composition of milk varied according to at least these two parameters. Richardson et al. (1961) found that low molecular weight fatty acids (C_6 to C_{12}) were depressed by approximately 2% at 85 F and 70% relative humidity. Increases of 6% and 4%, respectively, in the production of palmitic and stearic acids were obtained under the conditions noted above. Studies by Harper et al. (1961) on the volatile fatty acids in milk showed increases in fatty acid concentrations in pasteurized milk during storage. There were tenfold

increases in formic, acetic, butyric, valeric, caproic, and caprylic acids in pasteurized whole milk when it was stored for 21 days at 40 F. Propionic acid increased only twofold. The bacterial count of the milk after this storage period varied from 2.6×10^6 to 6.7×10^7 organisms per ml.

Botulinus poisoning is manifested in humans as a paralysis of the efferent branches of the autonomic nervous system. The specific sites of this paralytic action are the efferent parasympathetic nerves and/or the somatic nerves. Impulses transmitted across synapses are chemical in nature. The mechanism for synaptic transmission is composed of two subsystems one depending upon the adrenalin-like substance, sympathin, and the other on acetylcholine. Only the cholinergic system is affected by the botulinus toxin (Lamanna, 1959). The central nervous system is not involved. The effect upon the other tissues in the body is now known, although Lamanna (1959) speculated that tissue cultures of mammalian cells are not affected by the botulinus toxin since an organized cholinergic system is lacking.

The white mouse test is used almost exclusively for the detection of botulinus toxin; however, tissue culture monolayers have been employed to assay diphtheria and tetanus toxins (Rope et al., 1957). Gabliks and Solotorovsky (1961) used tissue culture derived from susceptible and resistant animals to detect diphtheria toxin. General cytotoxic experiments have been described by Eagle and Foley (1956) in which tissue culture was used to study the toxicity of carcinolytic material. Tissue culture techniques have been used to assay other metabolically produced bacterial products (Rightsel et al., 1958).

MATERIALS AND METHODS

Preparation of Spore Suspension and Counting Procedure

C. botulinum Types B and 62 A were used in all studies. The medium used to grow the culture for spore production consisted of 1.5% Trypticase (Difco), 1.0% Peptone (Difco), 0.5% sodium chloride, 0.25% potassium phosphate-di-basic, 0.3% glucose, 0.2% sodium thioglycollate and 0.0004% methylene blue chloride (Zoha and Sadoff, 1958). To obtain a spore crop, 200 ml of this medium were dispensed in a 1-liter Erlenmeyer flask fitted with a rubber stopper, equipped with a glass inlet tube projecting below the surface of the medium and with an outlet tube for flushing with natural gas. A magnetic stirring device was used to produce agitation. The outlet tube was connected to a trap containing a 1:1,000 dilution of aqueous quaternary ammonium compound to minimize the contamination of the atmosphere. One ml of a heat-shocked spore suspension of C. botulinum was added as inoculum.

Heat-shocking of the spores to stimulate germination was accomplished by placing the spore suspension in a water bath at 95 C for 30 min (Evans and Curran, 1943). To remove air from the culture medium, filtered natural gas was bubbled through the apparatus for approximately 30 min at which time a yellowish color became evident. Excess gas was burned off as it escaped from the outlet tube. The inlet tube and the outlet tube were clamped to maintain anaerobic conditions during incubation at 31 ± 1 C; after 7 days approximately 90% sporulation was observed. In preparing some spore inocula a medium consisting of 4% Trypticase and 1 ppm thiamine hydrochloride was

used (Day, 1960). Under these conditions sporulation was enhanced and approximately 95% sporulation occurred in 3 days.

The spores were harvested by centrifugation in a chilled Servall centrifuge at approximately 10,000 x g for 15 min. The spores were washed with Sorensen's phosphate buffer at pH 7.0 until the supernatant solution no longer showed a blue-green color due to the presence of residual methylene blue indicator. The stock spore suspension was stored in buffer (pH 7.0) in screw-cap glass bottles containing a few glass beads to facilitate mixing. The spore count of the stock suspension was 4.4×10^7 spores per ml. Little, if any, loss of viability in the spore suspension was observed on storage at 7 C for 17 months. In all instances the spore suspension was heat-shocked immediately before use.

All counts were made by diluting the samples in Sorensen's buffer solution. One ml of each dilution was added to each of 3 Prickett tubes containing 0.5 ml of a sodium thioglycollate-buffer solution made up as follows: 0.2% sodium thioglycollate, 0.2% potassium phosphate-di-basic and 0.1% sodium bicarbonate. Approximately 20 ml of a modified medium of Wynne et al. (1955), at 45 C, were added rapidly to each tube. Adequate mixing of the inoculated sodium thioglycollate-buffer solution and the medium was accomplished in this manner. This medium (YESB) consisted of 0.1% soluble starch, 1.0% yeast extract, 1.6% agar, and 0.0004% methylene blue chloride. After the medium solidified an additional 2 to 3 ml of sterile medium were added to maintain anaerobic conditions. Inoculation was undertaken at 37 C for 36 hr at which time colonies of C. botulinum were counted.

Toxicity Tests

Toxicity tests were performed to ascertain when toxin was present in the growth medium. The standard adult white mouse

toxicity test was used for the detection of botulinus toxin in the milk medium. When lethal levels of toxin were suspected a 0.5 ml sample of milk was injected into the peritoneal cavity of a white mouse. All mice were observed for 4 days following injection and those which survived were considered to have been inoculated with a nontoxic product. In most cases the test mice died within 24 to 36 hr. Inoculated animals which died within an hour after injection were considered to have died from traumatic injury and were eliminated from the experimental data. Mice which died in this manner were replaced with new animals so that at least 4 mice were used for each sample tested. Controls were inoculated with 0.5 ml of sterile skimmilk and skimmilk which had been heated to 95 C for 30 min. C. botulinum Type A antiserum was used in these tests to determine if the animals inoculated with the skimmilk containing toxin died due to botulinus poisoning. Equal volumes of antiserum (1:100) and skimmilk samples were incubated at room temperature for 10 min prior to injection.

Preparation of Skimmilk and Trypticase Media

Two hundred and eighty ml of raw, fresh skimmilk, sterilized at 121 C for 10 min, were added aseptically to each of 16, 12-oz screw-cap prescription bottles. One-liter quantities of skimmilk contained in 2-liter flasks were similarly sterilized and used as a reservoir to fill the bottles to within 5 ml of their maximum capacity. To determine if the skimmilk samples were sterile, duplicate 0.1 ml samples were plated on plate count agar and incubated at 37 C for 48 hr. Heat-shocked spores of C. botulinum Type 62 A and Type B were added to provide initial spore concentrations of approximately 10, 100, 1,000 and 10,000 spores per ml of skimmilk. Each sample was shaken vigorously for 1 min to assure mixing. One ml was removed for an initial or 0 hr

count and a sample of each spore concentration was incubated at 18, 13, 10 and 7 C. The temperature varied ± 1C. At 48 hr intervals each container was inverted 3 times and 1 ml of the skimmilk was removed for counting. Culture development was followed until the total count was in excess of one million cells per ml. Visual and olfactory observations were made on each sample.

To study the effect of oleic, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, and arachidic acids on the development of C. botulinum Type 62 A, 1% suspensions of the fatty acids were prepared in hot (90 to 95 C) buffer solution at pH 7. The mixtures were shaken until emulsions were formed. Each fatty acid emulsion was added to approximately 150 ml of fresh, raw skimmilk in 12-oz screw-cap bottles. In studies using skimmilk the fatty acid concentration was 0.01%. A mixture containing each fatty acid at a concentration of 0.001% was added to a sample; the total concentration of mixed acids was 0.01%. In addition the mixture of fatty acids was added to a skimmilk sample so that the concentration of mixed acids was 0.1%. A sample containing only skimmilk was maintained as a control. A bottle containing uninoculated skimmilk was maintained as a sterility control. All skimmilk-fatty acid combinations were sterilized by autoclaving at 121 C for 10 min. To completely fill these containers to approximately 375 ml, 225 ml of skimmilk previously sterilized in 1-liter volumes in 2-liter flasks, were added to each bottle. The skimmilk sterilized in the 1-liter volumes used to fill the storage bottles, was cooled rapidly in a water bath, pooled in a 4-liter flask and inoculated with heat-shocked spores of C. botulinum Type 62 A. The concentration of spores was 2×10^3 per ml. An air space of approximately 5 ml remained in each bottle. Samples were taken for bacterial cell counts at 0 hr and at 24 hr intervals. The counting medium was YESB medium and counts were made in Prickett tubes. Each bottle was inverted 3

times before sampling to adequately mix the skimmilk before sampling. Contaminants were tested for by transferring 1 ml of sample to 25 ml of Brain-Heart Infusion broth in a 25 x 150 mm test tube. The tubes were incubated at 37 C for 48 hr. One ml was transferred from each tube to a similar tube and incubated for an additional 48 hr. This transfer was necessary since the opacity of the skimmilk masked the turbidity produced by the growth of any contaminant which might have been present.

To determine the effect of agitation on the development of heat-shocked spores of C. botulinum Type 62 A, one series of samples was not shaken. Five bottles of each skimmilk-fatty acid mixture were prepared for this study.

To provide some basis for comparison a simple peptone medium, containing 4% Trypticase, was used in a series of experiments on the effect of fatty acids on culture development of C. botulinum Type 62 A. This peptone medium was selected as it represents a tryptic digest of casein. A 40% solution of Trypticase was prepared in distilled water and extracted 4 times with n-hexane (Skelly Solve B) to remove any fatty acids. Four hundred ml of the 40% solution of Trypticase were extracted with an equal volume of n-hexane. Each extraction lasted 15 min and was carried out in 2-liter flasks on a Burrell wrist-action shaker. The solvent was removed, pooled and fresh solvent added to the Trypticase solution. The solvent was evaporated and the residue resuspended in 10 ml of n-hexane.

Following extraction the 40% Trypticase solution was heated to 95 C for 10 min to remove residual solvent and a 4% Trypticase medium was prepared, containing 2.5% sodium thioglycollate-buffer solution. The buffer solution consisted of 4% sodium thioglycollate, 4% potassium phosphate-di-basic and 1% sodium bicarbonate. Culturing was undertaken in screw-cap test tubes rather than bottles to conserve medium;

since these samples were not agitated changing containers did not seriously affect the experimental design. Other studies have shown that the organism grew well under these conditions. A duplicate series of samples were set up in 12-oz bottles and were agitated. Each 12-oz screw-cap bottle received 375 ml of the Trypticase medium and each screw-cap tube received 28 ml of the medium. Fatty acids were tested at a concentration of 0.001%. All 10 fatty acids were tested in the bottles and only 4 acids were tested in screw-cap tubes (caprylic, capric, myristic, and stearic acids). Eight tubes of each fatty acid-Trypticase mixture were prepared. The fatty acid-Trypticase mixtures were sterilized by autoclaving for 10 min at 121 C. Each bottle and tube received heat-shocked spores of C. botulinum Type 62 A at a final spore concentration of 10^3 spores per ml. Incubation was at 18 C. Samples for bacterial counts were taken at 0 hr and at 24 hr intervals. Counting procedures were identical to the method previously described. Each bottle from the agitated series was inverted 3 times prior to sampling. The tubes were sampled by selecting a tube from each replicate set of 8, a 1 ml sample removed and the tube discarded. The remaining tubes within a replicate set were not disturbed.

To determine the effect of the extracted material from the 160 g of Trypticase the material was added to 375 ml of the 4% Trypticase medium in a 12-oz bottle. This sample was incubated at 18 C and agitated at the time of sampling. The initial spore concentration was 10^3 per ml.

Tissue Cell Culture Study

The use of tissue cell cultures for the detection of other bacterial toxins (Rope et al., 1957; Gabliks and Solotorovsky, 1961) encouraged the use of tissue cell cultures for the assay of purified toxin and toxin

produced in skimmilk. Lamanna (1959) proposed that tissue culture of mammalian cells would not be affected by botulinus toxin. In an effort to show that other mammalian tissues are affected by botulinus toxin and to develop a better test for the detection of the toxin, various tissue cultures were studied using cytotoxicity as the index of toxic action of the toxin of C. botulinum Type 62 A. Cell cultures of Mouse C_3H, Osgood J-111, Mouse L Cell, Embryo Monkey Kidney, Detroit-6-Parent and Detroit-6-Yale Clone were used in this study.[1] Based on their reproductive capacities each culture was planted at various initial concentrations; Mouse C_3H at 100,000 cells per ml, Embryo Monkey Kidney at 75,000 cells per ml, Detroit-6-Parent and Detroit-6-Yale Clone at 125,000 cells per ml, and Osgood J-111 and Mouse L Cell at 150,000 cells per ml. Cell stock suspensions were prepared by trypsinizing a monolayer cell sheet, washing the cells in cold buffer at pH 7, and replanting the cells in sterile glass bottles. These bottles were used as culture reservoirs. Cell concentrations were determined by microscopic examination of the cells stained with gentian violet in a Petroff-Hauser chamber. Cytoplasmic material was removed by adding citric acid to the staining preparation and the cell nuclei were counted.

Tissue cell cultures were transferred from the reservoir bottles and planted at cell concentrations as listed above in 25 ml plastic tissue culture flasks. Cultures were used when confluent monolayers were evident. The growth medium (Table 1) was removed and replaced with test medium. The test medium was composed of 10% horse serum (pretested for cytotoxicity) in Medium No. 199 (Cappell Laboratories). The medium contained phenol red as an acid-base indicator. The toxin

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[1]Obtained through the courtesy of the Virus Research Division, Parke, Davis and Co., Detroit, Michigan.

Table 1. Composition of Tissue Culture Growth Media

Culture Type	Media Composition
Embryo Monkey Kidney	Eagle's Tissue Culture Medium 15% Tryptose Phosphate Broth 10% Calf Serum
Mouse L Cell	Eagle's Tissue Culture Medium 20% Horse Serum
Osgood J-111	Eagle's Tissue Culture Medium 20% Human Serum
Detroit-6-Parent and Detroit-6-Yale Clone	Hank's Solution 40% Human Serum
Mouse C_3H	Eagle's Tissue Culture Medium 10% Human Serum

produced in the skimmilk was contained in the material harvested from the clear liquid portion of skimmilk remaining after incubation in the presence of C. botulinum Type 62 A at 18 C for greater than 90 days. Purified Type A toxin was also used.[1] The volume of the toxin produced in the skimmilk in each tissue culture flask was as follows: 1.0, 0.5, 0.25 and 0.1 ml. The purified toxin was diluted in a 1% saline solution so that the final concentration of toxin in the flasks was 1.0×10^{-5} (13 LD_{50}), 1.0×10^{-6} (1.3 LD_{50}), 2.0×10^{-6} (0.65 LD_{50}) and 5.0×10^{-6} ml (0.26 LD_{50}). The LD_{50} of the toxin produced in the skimmilk was calculated to be 0.000033 ml, and the LD_{50} of the purified toxin was calculated to be 0.00000075 ml. The method of Reed and Muench (1938) was used in calculating the LD_{50}. The volume of liquid in each flask was adjusted to 6 ml with medium and the pH was adjusted to pH 6.8 to 7.0 with a 1% solution of sodium bicarbonate. Duplicate controls of heat inactivated purified toxin, and heated toxin, produced in the skimmilk, (90 C for 30 min) were maintained. Cell controls of test medium in the presence of cells were also employed. Antiserum against the toxin was also used as a control at a dilution of 1:500. The skimmilk in which the toxin was produced was filtered through a bacterial retentive sintered glass filter to remove all cells.

All flasks were incubated at 37 C for 72 hr and observed for toxic action at 24 hr intervals. The tissue cultures were recorded photographically.

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[1]Obtained through the courtesy of the U. S. Army Biological Laboratory, Fort Detrick, Fredrick, Md.

RESULTS

Culture Development of C. botulinum Type 62 A in Skimmilk

The results obtained on the growth of C. botulinum Type 62 A in skimmilk are shown in Tables 2, 3, 4 and 5. Each value reported is an average of three replicate counts. A summary table showing the relationship of culture development of C. botulinum Type 62 A with toxin production, visual and olfactory changes in skimmilk is presented in Table 6.

Studies at 18 and 13 C. At 18 C the samples containing 10,000 heat-shocked spores per ml remained in the lag phase of culture development for approximately 9 days (Table 2). It was interesting to note that the total viable count decreased 90% during this period. Since the skimmilk was not heated prior to plating the total count obtained on each skimmilk sample represented vegetative cells and/or spores which may have germinated on the plating medium without heat-shocking at the time of plating. The decrease may be due to the failure of the spores to grow without heat-shocking or the failure of the spores to remain viable in the skimmilk after germination. After 8 days of storage reproduction was rapid and the cells attained a level of 4.9×10^7 per ml on the 22nd day. A slight amino odor, but no visible deterioration of the skimmilk, was detected after 10 days of storage. The amino odor became increasingly more pronounced on subsequent storage until a strong putrefactive odor was noted. This occurred after 26 days of storage and after the population attained a level of greater than 4.9×10^7 cells per ml. Although the identification of the amino odor was not attempted it might signify early utilization of the skimmilk

Table 2. Development of C. botulinum Type 62 A Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 18 C

Days of Storage	Approximate Initial Inoculum per ml			
	10	100	1,000	10,000
0[1]	7	64	800	5,500
2	4[2]	21[2]	100[2]	1,200[2]
4	3	8	100	800
6	1	3	43	530
8	1	2	40	730
10	1	1	33	2,200
12	1	1	50	5,100
14	1	2	37	1,500,000
16	1	1	5	2,600,000
18	1	1	9	6,600,000
20	1	1	5	2,600,000
22	1	1	14	49,000,000
24	13	32	38	--
26	430	800	1,000	--
28	530,000	1,000,000	1,600,000	--
30	4,300,000	7,300,000	8,500,000	--

[1]Zero hr counts represent spores.

[2]Represents the number of vegetative cells per ml in skimmilk.

substrate, since the odor was noted only in those cases when subsequent microbial growth occurred. Sterile skimmilk controls and inoculated skimmilk stored at inhibitory temperatures of 4 and 7 C did not contain any unusual odors. The presence of the amino odor was used, therefore, as a definite indication of metabolism of the skimmilk substrate by the microorganism. In all cases, deterioration of the skimmilk eventually occurred within 4 to 6 days after the amino odor was first noted (Table 6).

Toxicity tests performed after 14 days of incubation indicated possible toxin production since one of the 4 mice injected with this material died within 24 hr. On the 16th day of storage at 18 C the count was approximately 2.6×10^6 per ml, and all animals injected with this skimmilk died within 36 hr. Control mice injected with sterile skimmilk and mice receiving the inoculated 16-day-old skimmilk which had been heated, survived. On the 26th day of storage a strong putrefactive odor became evident. The first visual changes were observed after 28 days (Table 6).

Based on the exponential phase of culture development the generation time (g) was 13 hr, calculated by the method of Monod (1949). This figure is based on one trial using replicate tubes. There is a difference in the calculated generation time of 6 hr between the organisms inoculated at the 10,000 level and those inoculated at 1,000, 100 and 10 levels (Table 6). The limitation of the method of calculating the generation times was ± 2 hr. If a comparison is made between storage temperatures at an initial level some differences in the generation times can be seen. There is no significant difference between the generation time of the organisms at the 10,000 level at 18 C and the organisms at 10,000 level at 13 C (Table 6). Comparing the initial levels of 1,000, 100 and 10 at both temperatures, 18 C resulted in generation times of

7, 7 and 7 hr respectively, while 11, 11 and 8 hr were obtained, respectively, at 13 C. The greatest difference in the generation times was 4 hr. A shorter time can be expected at 18 C since it approximates more closely the optimum temperature for the organism (30 to 37 C), than does 13 C.

At 18 C with an initial concentration of approximately 1,000 spores per ml the duration of the lag phase was about 22 days (Table 2). This represents an increase of 13 days in the lag phase when compared to the lag obtained at an initial level of 10,000 cells per ml. There was a decrease of 99% in cell count during the lag phase when the initial inoculum was 1,000 cells per ml. A test for toxin was undertaken after 26 days of storage. All animals survived which indicated the absence of toxin in the skimmilk. The cell count was approximately 1,000 per ml and a slight amino odor was noted. After 28 days of incubation the viable cell count was approximately 1.6×10^6 per ml, and all test animals injected with this skimmilk died within 24 hr. Putrefactive changes were observed after 32 days of incubation. The generation time under these conditions was 7 hr.

At 18 C with initial inocula of 100 and 10 spores per ml the lag phases were 22 days in both cases (Table 2). In the former the count decreased more than 98%, and in the latter the decrease was approximately 86% during the lag phase. Rapid growth was initiated after 24 days of storage. All tests for toxin at both 24 and 26 days of incubation were negative. On the 28th day the cell counts were 1.0×10^6 and 5.3×10^5 per ml, respectively. All animals injected with this material died within 24 hr. No deaths were obtained in the animals injected with control skimmilk. This control material consisted of sterile and heated samples of stored skimmilk. Visual changes, exemplified by precipitation of the casein, and olfactory changes, recognized by a putrefactive odor characteristic of C. botulinum, were noted after 32 days of incubation. The generation time was 7 hr in both cases.

At the incubation temperature of 13 C the skimmilk inoculated with an initial inoculum of 10,000 spores per ml remained in the lag phase of culture development for 38 days (Table 3). A decrease of 97% in the bacterial count occurred during this period. Rapid growth of the organism occurred after 40 days of storage. An amino odor was noted after 42 days of incubation. The count was approximately 1.0×10^6 cells per ml on the 44th day. No toxin was present at this time. When the count reached 2.0×10^6 per ml on the 46th day of incubation, however, all animals injected with the skimmilk died within 24 hr. The characteristic odor of C. botulinum was noted after 48 days of storage, and the visual changes occurred 2 days later. The generation time based on the exponential phase of culture development was 14 hr. Although this generation time was slightly longer than the generation times calculated for the lower initial inocula at this storage temperature the difference was not appreciable when the degree of accuracy in calculating the generation times (± 2 hr) is considered.

When the initial inoculum of spores was 1,000 per ml and the incubation temperature was 13 C the lag phase was 44 days. A decrease of approximately 99% in the cell count was noted throughout this period. After 48 days of incubation rapid growth was observed and the population attained a level of 1.0×10^6 per ml. An amino odor was noted but toxicity tests proved negative at this time. After 50 days of storage however, all test animals injected with skimmilk died within 24 hr. No deaths were incurred in the control animals. Olfactory and visual changes, identical to those previously described, occurred on the 52nd and 54th day of incubation, respectively. The generation time was 11 hr.

At 13 C the organisms in the skimmilk at an initial level of 100 heat-shocked spores per ml exhibited a lag phase of 48 days (Table 3). A 96% decrease in cell count was noted during this period. Rapid growth

Table 3. Development of C. botulinum Type 62 A Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 13 C

Days of Storage	Approximate Initial Inoculum per ml			
	10	100	1,000	10,000
0[1]	6	67	900	5,300
2	7[2]	46[2]	570[2]	3,400[2]
4	5	24	240	1,700
6	2	20	160	1,200
8	3	23	250	1,200
10	2	18	270	1,300
12	1	9	230	670
14	1	9	67	470
16	1	9	80	300
18	1	5	47	730
20	1	3	84	200
22	1	3	29	370
24	1	10	17	300
26	1	26	24	300
28	1	14	18	330
30	1	34	11	230
32	1	41	11	170
34	1	34	19	270
36	1	33	22	230
38	1	27	24	300
40	1	23	20	600
42	1	33	62	--
44	1	42	63	1,000,000
46	1	38	1,000	2,000,000
48	1	63	1,000,000	8,000,000
50	1	1,000	2,200,000	--
52	70	950,000	8,200,000	--
54	1,000	2,800,000	--	--
56	1,400,000	6,800,000	--	--
58	6,300,000	--	--	--

[1]Zero hr counts represent spores.

[2]Represents number of vegetative cells per ml in skimmilk.

was initiated on the 48th day of incubation, and the cell count was approximately 63 per ml. Toxicity tests performed on a sample after 48 days proved negative. No amino odor was noted. After 50 days at 13 C the characteristic odor was evident. When the viable count reached 2.8×10^6 per ml, on the 54th day, all animals injected with skimmilk died within 24 hr. Animals injected with this skimmilk which had been heated survived. The putrefactive odor due to C. botulinum was noted on the 56th day of incubation with visual and olfactory changes occurring 2 days later. The generation time was 11 hr.

The lag phase of the culture, initially inoculated into the skimmilk at approximately 10 spores per ml and incubated at 13 C, was 50 days (Table 3). The decrease in count of the culture was approximately 83% during this prolonged lag phase. After 54 days of incubation the count attained a level of 1.0×10^3 cells per ml, and toxin was not present. A very slight amino odor was noted in the sample at this time. On the 56th day of storage 3 of 4 animals injected with the skimmilk died within 36 hr. After 58 days all test animals died within 24 hr. Olfactory and visual changes occurred on the 60th day of incubation. The generation time was 8 hr.

Studies at 10 and 7 C. The culture did not develop in the skimmilk inoculated with spores of C. botulinum at approximate initial levels of 10,000, 1,000, 100 and 10 per ml and stored at 10 and 7 C. A gradual decrease in cell count over the test period of 56 and 76 days, respectively, was noted (Tables 4 and 5). At 10 C the toxin assays carried out on the 7th, 14th, 28th and 56th day of incubation indicated that toxin was not present in the skimmilk. No visual or olfactory changes were noted at any time during the 56 days of the experiment (Table 6). At 7 C tests for toxin performed on the 14th, 26th, 40th and 76th day of incubation were negative. No visual or olfactory changes were noted at any time during the 76 day incubation period at 7 C (Table 6).

Table 4. Development of C. botulinum Type 62 A Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 10 C

Days of Storage	Approximate Initial Inoculum per ml			
	10	100	1,000	10,000
0[1]	5	56	600	5,400
2	6[2]	43[2]	430[2]	4,400[2]
4	7	42	400	3,300
6	4	35	270	3,200
8	3	31	500	4,300
10	2	24	130	2,600
12	2	23	100	2,700
14	2	24	100	1,000
16	2	18	230	1,700
18	2	19	200	1,300
20	2	12	130	6,000
22	3	13	200	4,000
24	1	17	100	4,600
25	1	19	100	4,000
26	2	19	100	1,300
28	1	18	100	1,300
30	1	12	100	1,000
32	1	14	83	1,300
34	2	13	90	1,000
36	1	5	83	1,000
38	1	5	70	1,000
40	1	4	67	1,000
56	1	10	100	1,000

[1]Zero hr counts represent spores.

[2]Represents number of vegetative cells per ml in skimmilk.

Table 5. Development of C. botulinum Type 62 A Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 7 C

Days of Storage	Approximate Initial Inoculum per ml			
	10	100	1,000	10,000
0[1]	10	73	800	5,200
2	8[2]	51[2]	500[2]	5,700[2]
4	6	36	300	4,500
6	5	43	190	2,300
8	5	21	280	5,100
10	4	40	380	5,700
14	4	36	330	2,700
16	4	27	500	2,600
18	5	29	330	3,700
24	3	30	150	1,000
26	4	24	130	730
28	2	21	60	1,900
34	3	16	100	1,500
38	3	15	130	2,000
40	2	15	110	1,300
42	4	16	120	1,500
44	4	16	120	1,700
46	6	16	100	1,500
74	3	8	60	790
76	4	7	60	600

[1]Zero hr counts represent spores.

[2]Represents number of vegetative cells per ml in skimmilk.

Table 6. Culture Development, Toxin Production, and Visual Changes in Skimmilk Inoculated with C. botulinum Type 62 A Spores and Incubated at Various Temperatures

Initial Inoculum (no./ml)	Duration of Lag Phase (days)	"g" Time (hr)	Decrease Before Growth (%)	Cell Conc. and Toxin Production (no./ml)	Time of Toxin Appear. (days)	Time of Changes (days)	
						Olfact.	Visual
			Storage Temperature 18 C				
10,000	9	13	90	2.7×10^6	16	26	28
1,000	22	7	99	1.6×10^6	28	32	32
100	22	7	98	1.0×10^6	28	32	32
10	22	7	86	5.3×10^5	28	32	32
			Storage Temperature 13 C				
10,000	38	14	97	2.0×10^6	46	48	50
1,000	44	11	99	2.2×10^6	50	52	54
100	48	11	96	2.8×10^6	54	56	58
10	50	8	83	1.4×10^6	56	60	60
			Storage Temperature 10 C				
10,000	56	--	--	NG[1]	NT[2]	NC[3]	NC
1,000	56	--	--	NG	NT	NC	NC
100	56	--	--	NG	NT	NC	NC
10	56	--	--	NG	NT	NC	NC
			Storage Temperature 7 C				
10,000	76	--	--	NG	NT	NC	NC
1,000	76	--	--	NG	NT	NC	NC
100	76	--	--	NG	NT	NC	NC
10	76	--	--	NG	NT	NC	NC

[1]No growth [2]No toxin [3]No change

Culture Development of C. botulinum Type B in Skimmilk

The data showing the effect of inoculating spores of C. botulinum Type B in skimmilk are presented in Tables 7, 8, 9 and 10. A summary table showing a comparison of Type B with Type 62 A is presented in Table 11.

Studies at 18 C. Heat-shocked spores of C. botulinum Type B inoculated into sterile skimmilk at an initial concentration of 1,000 spores per ml and stored at 18 C had a lag phase of 18 days (Table 7). The lag phase with Type B was 4 days shorter than the lag phase obtained with Type 62 A culture under similar conditions (Table 11). The maximum decrease of Type B culture during the lag phase was 70%. Tests for toxin production at the end of 18 days of incubation were negative and the bacterial population was 8.6×10^2 cells per ml. Growth was rapid on the 20th day of incubation and the bacterial count was 1.3×10^4 cells per ml. No toxin was present. On the 22nd day of incubation the population was 1.6×10^6 cells per ml and all animals injected with a sample of skimmilk died within 36 hr. No deaths were obtained in the control animals. Coagulation of the milk protein and the appearance of the putrefactive odor of Clostridium were evident on the 22nd day. A slight amino odor was noted one day prior to the appearance of the putrefactive odor. The generation time was 7 hr. The data on both types of cultures, inoculated into skimmilk at comparable levels and stored at 18 C, were generally in agreement. Type B culture produced a more rapid destruction of the substrate than did Type 62 A culture as visual and olfactory changes occurred 10 days earlier (Table 11). Type B culture produced toxin 6 days earlier than Type 62 A and had a shorter lag period.

At 18 C using a concentration of 100 spores per ml the culture remained in the lag phase for 30 days (Table 7). There was a difference

Table 7. Development of C. botulinum Type B Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 18 C

Days of Storage	Approximate Initial Inoculum per ml		
	10	100	1,000
0[1]	21	170	2,200
2	19[2]	180[2]	1,700[2]
4	15	180	1,700
6	14	200	1,500
8	15	130	1,400
10	11	130	1,400
12	11	120	1,300
14	9	110	830
16	7	77	660
18	8	87	860
20	4	40	13,000
22	4	53	1,600,000
23	6	71	--
24	6	84	--
26	6	120	--
28	6	120	--
30	3	140	--
32	4	110,000	--
34	4	3,000,000	--
36	5	6,600,000	--
38	4	--	--
40	5	--	--
56	5	--	--

[1]Zero hr counts represent spores.

[2]Represents the number of vegetative cells per ml in skimmilk.

of 8 days in the lag phases of Type B and Type 62 A at this level at 18 C. Type B culture had a longer lag phase. The decrease in total count during this lag phase (Type B) was 69%. Tests for toxin at the end of the lag phase were negative. After 32 days of storage toxin was present as 3 of 4 animals injected with skimmilk died within 48 hr. The count at this time was 1.1×10^5 cells per ml. On the 34th day of incubation the count was 3.0×10^6 cells per ml and all mice died in 24 hr. No deaths were obtained in the control animals. A slight amino odor was noted after 32 days of incubation, and the strong putrefactive odor of Clostridium was present after 34 days of storage. Visual changes were noted on the same day. The generation time was 10 hr. The data obtained with both types of cultures, inoculated at 100 spores per ml and stored at 18 C, agreed fairly well. Type 62 A culture had a generation time of 7 hr, and Type B had a generation time of 10 hr. The lag phase was 8 days longer with Type B culture. The generation time was not appreciably longer with Type B culture. At the 100 per ml initial level the production of visual and olfactory changes became evident at the same time (Table 11).

When the initial level of spores of C. botulinum Type B was 10 per ml and the incubation was at 18 C a decrease of 85% in cell count was noted over the test period of 56 days (Table 7). No growth, toxin production, visual or olfactory changes were noted at any time. Type 62 A culture at comparable levels and incubation temperature brought about visual and olfactory changes of the skimmilk in 32 days (Table 11).

Studies at 13, 10 and 7 C. With initial inocula of 10,000, 1,000, 100 and 10 per ml in sterile skimmilk a gradual decrease in cell population was observed over the test period of 56 days (Tables 8, 9 and 10). The storage temperatures of 13, 10 and 7 C did not permit the growth of C. botulinum Type B. No toxin was produced nor was there any evidence of visual or olfactory changes during this prolonged

Table 8. Development of C. botulinum Type B Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 13 C

Days of Storage	Approximate Initial Inoculum per ml			
	10	100	1,000	10,000
0[1]	20	183	2,200	16,000
2	16[2]	200[2]	1,930[2]	21,000[2]
4	15	170	1,800	12,000
6	16	190	1,760	12,000
8	15	170	1,430	16,000
10	11	180	1,760	13,000
12	11	170	1,470	11,000
14	10	190	1,330	12,000
16	9	200	660	14,000
18	11	180	630	12,000
20	7	77	570	6,600
22	8	70	630	5,300
23	4	76	430	9,300
24	4	84	430	9,000
26	6	88	460	6,300
28	4	80	530	6,300
30	3	82	330	2,300
32	3	93	260	2,300
34	3	83	360	2,700
36	2	42	160	2,000
38	2	51	230	1,700
40	2	37	200	2,000
56	1	28	100	2,300

[1]Zero hr counts represent spores.

[2]Represents the number of vegetative cells per ml in skimmilk.

Table 9. Development of C. botulinum Type B Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 10 C

Days of Storage	Approximate Initial Inoculum per ml			
	10	100	1,000	10,000
0[1]	24	160	3,000	16,000
2	16[2]	190[2]	1,800[2]	18,000[2]
4	19	150	1,900	17,000
6	18	160	1,500	20,000
8	16	150	2,000	19,000
10	16	160	1,500	20,000
12	15	160	1,500	20,000
14	17	150	1,300	18,000
16	15	120	1,300	15,000
18	17	140	1,100	16,000
20	13	130	1,000	16,000
22	13	120	1,000	16,000
23	11	94	1,200	13,000
24	11	93	1,300	11,000
26	13	120	1,000	14,000
28	13	120	900	11,000
30	9	120	1,400	9,300
32	10	110	1,400	9,300
34	8	110	1,500	8,300
36	10	69	900	12,000
38	10	72	1,100	10,000
40	8	78	1,100	9,000
56	5	34	500	9,300

[1]Zero hr counts represent spores.

[2]Represents the number of vegetative cells per ml in skimmilk.

Table 10. Development of C. botulinum Type B Spores Inoculated Into Skimmilk at Different Concentrations and Incubated at 7 C

Days of Storage	Approximate Initial Inoculum per ml			
	10	100	1,000	10,000
0[1]	20	180	1,960	15,000
2	17[2]	190[2]	1,800[2]	16,000[2]
4	19	150	1,600	15,000
6	17	120	1,800	15,000
8	15	140	1,700	15,000
10	15	170	1,500	17,000
12	16	180	1,500	17,000
14	15	130	1,900	18,000
16	13	140	1,100	14,000
18	14	120	1,000	14,000
20	13	93	1,300	14,000
22	13	97	1,300	12,000
23	11	100	1,700	11,000
24	12	100	1,200	10,000
26	9	120	1,200	10,000
28	10	120	1,200	11,000
30	6	110	870	9,000
32	7	98	800	8,600
34	8	120	860	6,000
36	8	100	960	6,000
38	6	91	830	4,300
40	7	94	1,000	6,300
56	8	50	430	5,300

[1]Zero hr counts represent spores.

[2]Represents the number of vegetative cells per ml in skimmilk.

Table 11. Comparison of the Culture Development of C. botulinum Types 62 A and B Spores Inoculated Into Skimmilk at Various Levels and Incubated at Various Temperatures

Storage Temperature	Initial Inoculum (no./ml)	Duration of Lag Phase		"g" Time		Time of Toxin Prod.		Time of Changes: Visual		Time of Changes: Olfact.	
		62A	B	62A	B	62A	B	62A	B	62A	B
		(days)		(hr)		(days)		(days)			
18 C	1,000	22	18	7	7	28	22	32	22	32	22
	100	22	30	7	10	28	32	32	34	32	34
	10	22	>56	7	NG[1]	28	NT[2]	32	NC[3]	32	NC
13 C	10,000	38	>56	14	NG	46	NT	48	NC	50	NC
	1,000	42	>56	11	NG	50	NT	52	NC	54	NC
	100	48	>56	11	NG	54	NT	56	NC	58	NC
	10	50	>56	8	NG	56	NT	60	NC	60	NC
10C	10,000	>56	>56	NG	NG	NT	NT	NC	NC	NC	NC
	1,000	>56	>56	NG	NG	NT	NT	NC	NC	NC	NC
	100	>56	>56	NG	NG	NT	NT	NC	NC	NC	NC
	10	>56	>56	NG	NG	NT	NT	NC	NC	NC	NC
7 C	10,000	>76	>76	NG	NG	NT	NT	NC	NC	NC	NC
	1,000	>76	>76	NG	NG	NT	NT	NC	NC	NC	NC
	100	>76	>76	NG	NG	NT	NT	NC	NC	NC	NC
	10	>76	>76	NG	NG	NT	NT	NC	NC	NC	NC

[1]No growth

[2]No toxin

[3]No change

incubation period. At 13 C the cell populations decreased 89, 95, 86 and 95% when the initial spore levels were 10,000, 1,000, 100 and 10, respectively. At incubation temperature of 10 C the decreases in cell counts of all the initial levels were approximately 82%. Similarly, at 7 C the decreases in cell counts were approximately 75%.

In summary, at 18 C C. botulinum Type B grew, produced toxin, visual and olfactory changes characteristic of Clostridium when inoculated into sterile skimmilk at levels of 1,000 and 100 per ml (Tables 7 and 11). At this storage temperature an initial inoculum of 10 spores per ml did not develop into an active vegetative culture within 56 days. Regardless of the initial inoculum, incubation at 13, 10 and 7 C did not permit the growth of the organism. When the increase of bacterial population became evident a slight amino odor was present in all cases immediately before the appearance of toxin. Toxin could not be found in the skimmilk at the time of the appearance of the amino odor. The appearance of the amino odor immediately before toxin production corresponds with the reaction of Type 62 A culture in skimmilk.

Effect of Fatty Acids in Skimmilk on Culture Development

The data showing the effect of various fatty acids, at a concentration of 0.01%, in skimmilk, on the culture development of spores of C. botulinum Type 62 A are presented in Tables 12 and 13.

Culture development in agitated skimmilk. Spores of C. botulinum developed in skimmilk and in 22 days the cells attained a level of 9.3×10^5 per ml (Table 12). The lag phase of the culture in this skimmilk sample, which did not contain any fatty acids, was 20 days. Butyric, caproic, arachidic, and oleic acids exerted little if any effect on the lag phase of culture development. In the presence of caprylic, lauric, and palmitic acids the lag phases were 24 days as compared

Table 12. Effect of Various Fatty Acids (0.01%) in Skimmilk on the Germination and Outgrowth of Spores of C. botulinum Type 62 A--Samples Shaken

Days of Incubation at 18 C	Control	Fatty Acids				
		Butyric	Arachidic	Oleic	Caproic	Caprylic (no./ml x 100)
0[3]	18.0	18.0	18.0	18.0	18.0	18.0
3	18.0	12.0	16.0	11.0	14.0	16.0
6	1.3	1.8	1.0	1.1	1.1	1.2
9	.55	.7	.7	.8	.5	.5
12	.35	.4	.3	.4	.2	.2
15	.30	.3	.3	.3	.2	.3
18	.20	.1	.2	.1	.2	<.1
19	.15	.1	.1	<.1	.1	<.1
20	.15	.1	.1	.1	.1	.1
21	6.0	.4	.7	.6	<.1	.1
22	9300.0	10.0	40.0	120.0	<.1	<.1
23	--	25000.0	32000.0	61000.0	.1	.1
24	--	--	--	--	1.5	.1
25	--	--	--	--	7000.0	6.5
26	--	--	--	--	31000.0	9000.0
27	--	--	--	--	--	--
28	--	--	--	--	--	--
32	--	--	--	--	--	--

[1]Mixture of all acids, each acid at 0.001% (total acid concentration 0.01%).

[2]Mixture of all acids, each acid at 0.01% (total acid concentration 0.1%).

[3]Zero hr counts represent spores.

Palmitic	Lauric	Myristic	Capric	Stearic	A.A.M.[1]	A.A.E.[2]
18.0	18.0	18.0	18.0	18.0	18.0	18.0
14.0	18.0	12.0	17.0	14.0	18.0	11.0
1.7	1.1	1.4	1.5	1.6	1.3	1.4
.4	.8	.5	.6	.5	.6	.6
.2	.3	.5	.4	.3	.2	.3
.1	.4	.4	.3	.2	.1	.3
<.1	.3	.2	.2	<.1	.2	.2
<.1	.2	.3	.2	<.1	<.1	.1
.1	.1	.2	.2	<.1	<.1	.2
.2	.2	.1	.1	.1	.1	.1
.3	.1	.2	.1	.1	<.1	<.1
.2	<.1	.2	.1	.1	<.1	<.1
.9	.4	.1	.1	<.1	<.1	<.1
4100.0	1200.0	.1	.1	.1	<.1	<.1
60000.0	40000.0	.1	.2	.1	<.1	<.1
--	--	4.7	5.2	6.0	.1	<.1
--	--	>10000.0	>10000.0	>10000.0	.1	<.1
--	--	--	--	--	.1	.1

with the 20 days lag period obtained in the control. Rapid growth of the culture in the presence of these acids was initiated after 24 days of incubation at 18 C. Myristic, capric, and stearic acids extended the lag phase of culture development of C. botulinum Type 62 A in skimmilk 6 days. This extension of the lag phase for 6 days is an appreciable increase over the 20 day lag phase of the culture in the control skimmilk medium.

A mixture of all 10 fatty acids in skimmilk, each at a concentration of 0.001%, completely inhibited culture development over the test period of 32 days. When each fatty acid was added to skimmilk at a concentration of 0.01% (total concentration of acid was 0.1%) growth was also completely inhibited during the test period. It is apparent that certain individual fatty acids were inhibitory to the development of the culture, but the mixtures of all acids were more effective. Althouth each of the 3 most inhibitory acids at a concentration of 0.01% extended the lag phase 6 days, the mixtures of acids consisting of individual acids at a concentration of 0.001%, increased the lag phase for periods of time greater than 12 days (Table 12). A mixture of myristic, capric, and stearic acids each at 0.001% may have been as effective in prolonging the lag phase of culture development. Although sampling was terminated after 32 days no physical and/or olfactory changes were evident in the skimmilk after 45 days at 18 C. This indicated that the mixtures of all 10 acids, each at one-tenth the concentration at which the individual acids were tested, gave greater inhibitory action.

Culture development in unagitated skimmilk. The study on unagitated medium was devised to determine the effect of introducing air into the container at the time of sampling. Results showing the effect of fatty acids on culture development in unagitated skimmilk are presented in Table 13. In skimmilk the lag phase of culture development, determined from the growth curve, of C. botulinum Type 62 A was approximately 15 days. Of the four acids tested, caprylic acid had the least effect and the lag phase of the culture in the presence of this acid at 0.01% concentration

Table 13. Effect of Various Fatty Acids (0.01%) in Skimmilk on the Germination and Outgrowth of Spores of C. botulinum Type 62 A--Samples Not Shaken

Days of Incubation at 18 C	Control	Fatty Acids					
		Caprylic	Capric	Myristic	Stearic	A.A.M.[1]	A.A.E.[2]
		(no./ml x 100)					
0[3]	18.0	18.0	18.0	18.0	18.0	18.0	18.0
3	14.0	13.0	16.0	12.0	14.0	14.0	8.0
10	1.5	1.1	.9	1.2	1.0	1.2	1.1
16	9.0	.4	.4	.3	.3	.2	.3
17	6200.0	--	--	--	--	--	--
18	80000.0	--	--	--	--	--	--
21	--	.3	.1	.2	.3	.2	.2
23	--	1.1	.1	.2	.2	.1	.2
24	--	4100.0	7.0	4.1	.2	.1	.2
25	--	40000.0	3100.0	5000.0	6.9	<.1	<.1
26	--	--	42000.0	61000.0	9600.0	<.1	<.1
40	--	--	--	--	--	<.1	<.1

[1]Mixture of all acids, each acid at 0.001% (total acid concentration 0.01%).

[2]Mixture of all acids, each acid at 0.01% (total acid concentration 0.1%).

[3]Zero hr counts represent spores.

was 22 days. This represents an increase of 7 days in the lag phase beyond the control. Capric and myristic acids inhibited culture development for 23 days, and stearic acid extended the lag phase to 24 days (Table 13). The mixture of acids, at both concentrations, inhibited culture development for the entire test period. No growth was present in the unagitated skimmilk after 40 days of incubation at 18 C (Table 13).

Effect of Fatty Acids in Trypticase Medium on Culture Development

The effect of various fatty acids, in a 4% Trypticase medium on the culture development of C. botulinum Type 62 A is presented in Tables 14 and 15. The fatty acids were tested at a concentration of 0.001% in contrast to the previous study in which the concentration of fatty acids was 0.01%.

Culture development in agitated Trypticase medium. Butyric, caproic, lauric, arachidic, palmitic and oleic acids did not inhibit the growth of the culture. The lag phase of culture development in the extracted Trypticase medium, the unextracted medium, and the presence of these 5 fatty acids was 3 days. The lag period of the culture was 5 days in the presence of caprylic and capric acids, and 6 days in the presence of myristic and stearic acids. The mixture of all 10 fatty acids, at a concentration of 0.001%, extended the lag phase for 3 days, and the acid mixture with each acid at 0.001% (total concentration of acid was 0.01%) extended the lag phase for 4 days. The lag phases were 6 and 7 days, respectively. The material which was extracted from the 40% Trypticase solution and tested in the 4% Trypticase medium, extended the lag period for 3 days. The lag phase was 6 days.

Culture development in unagitated Trypticase. Caprylic, capric, myristic, and stearic acids were tested in this study. In the control

Table 14. Effect of Various Farry Acids (0.001%) in 4% Trypticase on the Germination and Outgrowth of Spores of C. botulinum Type 62 A--Samples Shaken

Days of Incubation at 18 C	Control[1]	Control[2]	Control[3]	Fatty Acids		
				Butyric	Caproic	Lauric
				(no./ml x 100)		
0[6]	9.8	8.7	9.5	11.0	10.0	12.0
1	9.0	6.9	7.0	9.0	8.2	10.0
2	8.1	7.4	6.2	7.7	8.0	7.5
3	6.8	5.2	4.4	7.1	6.8	6.8
4	6,500.0	1,200.0	4.1	110.0	9.7	480.0
5	>100,000.0	>100,000.0	5.0	>100,000.0	9,300.0	64,000.0
6	--	--	6.6	--	--	--
7	--	--	2,400.0	--	--	--
8	--	--	--	--	--	--
9	--	--	--	--	--	--

[1]Hexane-extracted Trypticase

[2]Unextracted Trypticase

[3]Trypticase extracted with hexane plus soluble material from Trypticase.

[4]Mixture of all acids each at 0.0001% (total acid concentration 0.001%).

[5]Mixture of all acids each at 0.001% (total acid concentration 0.01%).

[6]Zero hr counts represent spores.

Oleic	Arachidic	Palmitic	Stea
9.7	9.6	9.9	
9.1	9.5	9.3	
8.4	8.1	8.1	
6.1	4.9	8.0	
970.0	9.1	1,000.0	
>100,000.0	16,000.0	>100,000.0	
--	--	--	
--	--	--	26,
--	--	--	
--	--	--	

sample of extracted 4% Trypticase medium the culture developed in 3 days (Table 15). The lag phase of the culture in the unextracted Trypticase medium was also 3 days. The lag phase was 5 days in the presence of capric and myristic acids. Caprylic and stearic acids prolonged the period of culture development; the lag period was 6 days. The mixtures of acids, at concentration of 0.001% and 0.01%, affected the development of the culture; the lag phases were 6 and 7 days, respectively.

Effect of Botulinus Toxin on Various Tissue Culture Monolayers

The results of this study are found in Table 16 and in Figures 1, 2, 3 and 4. With the exception of Mouse L Cell culture all cultures tested were found to be adversely affected by botulinus toxin (Figure 1, Table 16). The most sensitive cultures were Detroit-6-Parent and Detroit-6-Yale Clone. The cytotoxicity in the sensitive cultures was essentially the same in all cases. The confluent monolayer of cells was disrupted with the appearance of intracellular spaces. In all cases of sensitivity early cytotoxicity of the monolayer occurred after 24 hr of incubation at 37 C. Further degeneration of the cells occurred, exhibited by granulation and shrinking of the cytoplasmic mass and of the nucleus. Long slender streams of cytoplasmic material connected masses of condensed cells (Figure 2F). In the final stages of degeneration the cells were reduced to tightly packed masses of cells. At the end of the test period of 72 hr a few cells remained unaffected. Generally, the cytopathology exhibited by the cells was that of old, naturally deteriorating cells in culture.

The 72 hr test period was selected since cells maintained in culture without a change of medium begin to show some slight deterioration after storage for extended periods of time. After 72 hr the pH of

Table 15. Effect of Various Fatty Acids (0.001%) in 4% Trypticase on the Germination and Outgrowth of Spores of C. botulinum Type 62 A--Samples Not Shaken

Days of Incubation at 18 C	Control[1]	Control[2]	Fatty Acids					
			Capric	Myristic	Stearic	Caprylic	A.A.M.[3]	A.A.E.[4]
			(no./ml x 100)					
0[5]	9.6	11.0	9.0	9.8	9.8	10.0	9.2	9.6
1	8.1	7.7	8.6	9.1	6.9	7.9	8.4	9.0
2	8.0	7.2	6.2	5.8	5.5	6.0	7.0	7.2
3	7.8	7.4	4.1	5.0	4.9	5.2	6.6	4.2
4	16,000.0	21,000.0	3.8	2.9	4.0	4.6	5.1	4.0
5	--	--	4.0	2.6	4.1	5.0	4.8	3.6
6	--	--	2,600.0	6,100.0	5.0	6.9	3.1	4.0
7	--	--	>100,000.0	>100,000.0	11,000.0	9,600.0	1,100.0	4.1
8	--	--	--	--	--	--	140,000.0	100.0
9	--	--	--	--	--	--	--	590,000.0

[1]Hexane-extracted Trypticase.

[2]Unextracted Trypticase.

[3]Mixture of all acids each at 0.0001% (total acid concentration 0.001%).

[4]Mixture of all acids each at 0.001% (total acid concentration 0.01%).

[5]Zero hr counts represent spores.

Table 16. Effect of Varying Levels of Botulinus Toxin on Tissue Culture Monolayers

Type of Cell Culture	Hours of Incubation	Cytotoxic Effect							
						Volume of Milk Toxin			
		C.C.[1]	T.C.[2]	A.C.[3]	P.T.[4]	0.1	0.25	0.5	1.0
Mouse C_3H	12	0	0	--	--	0	0	0	0
	24	0	0	--	--	0	0	0	0
	48	0	0	--	--	0	0	0	0
	72	+	++	--	--	+++	++++	++++	++++
Osgood J-111	12	0	0	--	--	0	0	0	0
	24	0	0	--	--	0	±	+	+
	48	0	0	--	--	±	+	++	++
	72	0	0	--	--	+	+	++	+++
Mouse L Cell	12	0	0	--	--	0	0	0	0
	24	0	0	--	--	0	0	0	0
	48	0	0	--	--	0	0	0	0
	72	±	0	--	--	0	0	0	0
Detroit-6-Parent	12	0	0	--	--	0	0	0	0
	24	0	0	--	--	0	±	++	+++
	48	0	0	--	--	0	+	+++	++++
	72	0	+	--	--	+	++	++++	++++
Embryo Monkey Kidney	12	0	0	0	0	0	0	0	0
	24	0	0	0	+	0	0	0	+
	48	0	0	0	++++	0	0	+	++
	72	0	0	0	++++	0	±	++	++++
Detroit-6-Yale Clone	12	0	0	0	0	0	0	0	0
	24	0	0	0	++	0	+	++	+++
	48	0	+	0	++++	±	++	++	++++
	72	0	+	0	++++	+	+++	+++	++++

0 = no cytotoxicity; ++++ = maximum cytotoxicity; -- = not tested

[1]Cell control contained only growth medium.

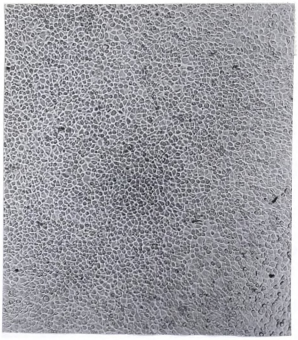
[2]Toxin control contained growth medium and 1 ml of heat inactivated milk toxin.

[3]Antiserum control contained growth medium, purified toxin and 1 ml antiserum 1:500.

[4]Purified toxin.

the test medium became acid, as indicated by the change in color of the phenol red indicator from red to yellow. Since the production of acid by metabolizing cells in culture is normally expected no significance was attributed to this change. A complete or partial change of the test medium would have prevented any change in the cells. The medium was not changed since the flasks which contained the toxin did not receive a change of medium.

When the medium was harvested from the culture flasks after the test period, and injected into white mice, the toxin titer was not decreased. All mice injected with the unheated toxin produced in skim-milk died within 36 hr, while those mice receiving the same material which was heated lived. The toxin and antiserum mixtures from the test flasks were not toxic to the mice.



A- Heat inactivated botulinus toxin (1.0 ml) 72 hr

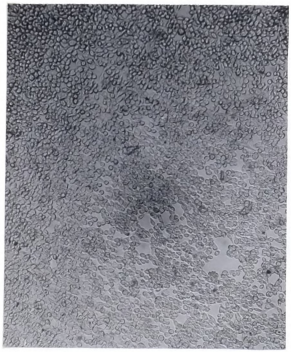
B-Cell control 72 hr

C-Botulinus toxin (0.1 ml) 72 hr

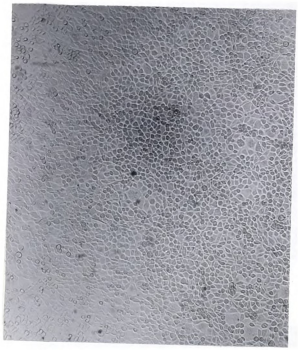
D-Botulinus toxin (0.25 ml) 72 hr

Figure 1. Botulinus toxin in Mouse L Cell tissue culture.

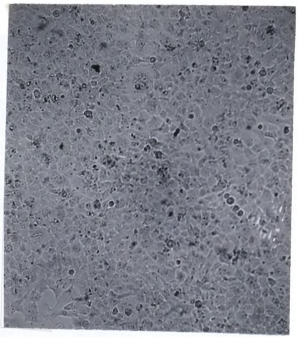
Figure 1 - continued



E-Botulinus toxin
(0.5 ml) 72 hr



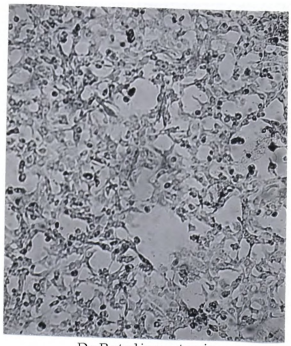
F-Botulinus toxin
(1.0 ml) 72 hr



A- Heat inactivated toxin
(1.0 ml) 72 hr

B-Cell control
72 hr

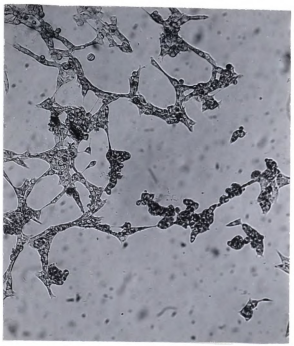
C-Botulinus toxin
(0.1 ml) 72 hr



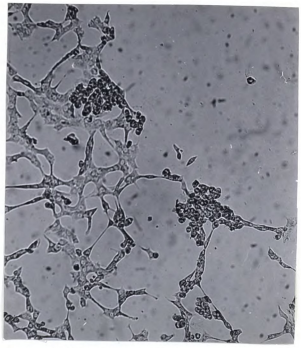
D-Botulinus toxin
(0.25 ml) 72 hr

Figure 2. Effect of botulinus toxin on Detroit-6-Parent tissue culture.

Figure 2 - continued



E-Botulinus toxin
(0.5 ml) 72 hr



F- Botulinus toxin
(1.0 ml) 72 hr

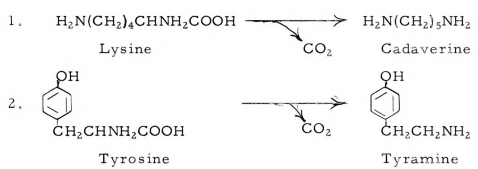
DISCUSSION

Recent developments in ultra-high temperature pasteurization (UHT) have increased the storage life of milk but have not resulted in sterility (Tobias et al., 1955; Speck, 1961). Although sterility is not assured there is evidence of a state of "practical sterility" being established. This term describes a state where not all bacterial spores are killed but the survivors are unable to grow and cause spoilage due to a number of factors: 1) degree of anaerobiosis in the container, 2) storage temperature, and 3) the formation during sterilization of various compounds inhibitory to spore germination. The increased storage life of this nonsterile food might present a public health problem if the heat resistant spores of C. botulinum survived the UHT pasteurization or if the spores present as post-pasteurization contaminants overcame these inhibitory factors and germinated.

This study has shown that heat-shocked spores of C. botulinum Types 62 A and B were able to germinate and grow in sterile skimmilk when stored at relatively low temperatures. Conditions for germination and growth varied slightly between the two types. The Type 62 A culture was able to grow and produce toxin in sterile skimmilk at all inoculum levels when the temperature of incubation was 13 or 18 C. Type B grew in sterile skimmilk only at 18 C, and only when the initial inoculum level of heat-shocked spores was 100 per ml or greater. Lower inoculum levels of Type B at temperatures below 18 C resulted in no growth, no toxin production, and no physical or olfactory changes.

Invariably a slight, but distinctly noticeable, amino odor immediately preceded the appearance of botulinus toxin in the skimmilk medium. No toxin was evident when the amino odor first became

noticeable. Some speculation may be made at this time as to the origin and eventual fate of the compound causing this odor. Since the amino odor occurred in the skimmilk medium at the beginning of active multiplication, it can be assumed that the odor indicated the early proteolytic activities of the culture. No such odor occurred in control samples of skimmilk in which the organism failed to grow or in sterile skimmilk stored for long periods of time. The amines present in the medium due to the early proteolytic activities might constitute a variety of structures depending upon the amino acid source. Since skimmilk proteins are composed of a variety of amino acids the identification of the source amino acid would be very difficult. The presence of the amines could be attributed to a number of factors. Among these could be included the early degradation of casein into shorter units and the subsequent decarboxylation of the amino acids into amines. The amines could be detected by their characteristic odor in more advanced stages of putrefaction as part of the putrefactive odor of Clostridium. Typical reactions can be illustrated as follows:



Both cadaverine and tyramine have very distinctive amino odors.

All members of the genus Clostridium display an active amino acid metabolism. The nutritional requirements of organisms vary considerably and it has been shown (Nisman, 1954) that the principal chemical reaction by which C. botulinum obtains energy is coupled

deamination. This occurs when the culture is grown on amino acids as the sole source of carbon and nitrogen. The reaction, called the Stickland Reaction, is described as a coupled deamination between two amino acids where one is the donor and the other the acceptor of protons. The reaction can be illustrated as follows:

1. $CH_3CHNH_2COOH + 2NH_2CH_2COOH + 2H_2O \longrightarrow 3CH_3COOH + 3NH_3 + CO_2$

   Alanine Glycine

2. $CH_3CHNH_2COOH + 2H_2C(CH_2)CHCOOH(NH) + 2H_2O \longrightarrow 2NH_2(CH_2)_4COOH + CH_3COOH + NH_3 + CO_2$

   Alanine Proline

Milk constituents, which are water, protein, fat, carbohydrate, inorganic salts, and vitamins, can be placed into 3 groups on the basis of their solubilities: 1) some are in true solution, 2) some are partly in solution and partly in suspension or colloidal suspension, and 3) some are present entirely in the colloidal state. Casein, which is the principal protein of milk, is present in milk entirely in the colloidal state and can be utilized for growth and reproduction by C. botulinum. The utilization of the protein is accomplished through hydrolysis followed by the Stickland Reaction, under anaerobic conditions and in the presence of cofactors. Milk contains not only casein, which can be hydrolyzed into its component amino acids, but also some free amino acids which can be readily utilized by the organism in the Stickland Reaction.

Although this study has shown that C. botulinum Types 62 A and B can grow and produce toxin in skimmilk it must be pointed out that this study, carried out in experimentally inoculated sterile skimmilk, investigated the natural development of the pure culture of C. botulinum.

The observation that toxin was produced in certain instances under these experimental conditions does not necessarily imply that the presence of this organism in pasteurized skimmilk poses a health hazard. The assumption that C. botulinum spores would constitute the only microbial contaminant in milk is possible but unlikely. Undoubtedly other sporeforming or nonsporeforming bacteria would be associated with the contaminants responsible for the presence of Clostridium. The development of Clostridium in association with other bacteria, a condition more truly representative of nature, may considerably alter culture development and/or toxin production. The development of Staphylococcus aureus (Oberhofer and Frazier, 1961) is affected when grown in the presence of other organisms. Likewise, toxin production by C. botulinum (Grecz et al., 1959a) is reduced in the presence of other organisms. With respect to the possibility of C. botulinum becoming a health hazard in milk, the incubation temperature of milk today and its storage life have not approached the experimental conditions of this investigation. But as was previously stated, with the advent of new processes of pasteurization (UHT), longer storage periods at higher temperatures can be expected. As these factors approach the experimental conditions of this investigation, a health hazard, due to growth of C. botulinum in skimmilk or whole milk, might become a reality.

Literature reports on the inhibition of culture development of C. botulinum have emphasized complete inhibition of growth by certain fatty acids. Foster and Wynne (1948) reported that linoleic and linolenic acids at 0.1% concentration inhibited growth of C. botulinum. Roth and Halvorson (1952) have inhibited spore germination of C. botulinum using 0.1% rancid fatty acids. Grecz et al. (1959a) have found that in cheese a 2% concentration of mixed fatty acids became

the inhibitory level for C. botulinum. In the present study on the effect of fatty acids on the culture development of heat-shocked spores of C. botulinum Type 62 A individual fatty acids had a slight effect in partially inhibiting culture development, but a composite mixture of the 10 fatty acids appreciably extended the lag phase of culture development. In skimmilk the composite mixtures of fatty acids at 0.1% and 0.01% completely inhibited culture development of C. botulinum while in a 4% Trypticase medium the fatty acid mixture at 0.01% and 0.001% merely extended the lag phase by approximately 4 and 3 days, respectively. In all cases of partial inhibition of culture development the mixtures of fatty acids were more effective than any individual fatty acid at comparable concentrations. Mixtures of fatty acids more closely represent their natural distribution in milk. The concentration and type of fatty acid would be important in controlling the metabolic activities of C. botulinum in milk. Since the inhibitory effect on the culture is enhanced by increasing the spectra of acids it becomes evident that fatty acids are at least one of the constituents of milk which can partly inhibit the culture development of heat-shocked spores of C. botulinum Type 62 A.

Krukovsky (1961) has shown a high lipase activity in milk before pasteurization. Hilditch and Longenecker (1937) made some attempts to characterize the component fatty acids in milk but not until recently have Harper et al. (1961) made some critical analyses on the concentration of free fatty acids in milk. Their investigation was on the free volatile fatty acids in milk and they have found that butyric, caproic and caprylic acids, among others, were present. These three fatty acids were included in the present investigation. Although additional evidence of the presence of higher molecular weight fatty acid constituents of milk was not found in the literature it is not too unrealistic to expect the presence of these higher molecular species of acids in milk.

A comparison was made of the lag phases of the culture in agitated (sample inverted three times prior to sampling) skimmilk versus unagitated skimmilk (Table 17). The lag period of the culture in unagitated skimmilk was 5 days shorter than the lag phase in the agitated skimmilk. The introduction of air into the storage container during sampling appreciably extended the lag phase of culture development of C. botulinum. A difference of at least 2 days between the lag phases was considered to be significant, whereas ± 1 day was considered to be within the limits of error in determining the lag phases. The lag periods of the culture in skimmilk containing capric, caprylic, myristic, and stearic acids were 3, 2, 3, and 2 days shorter than the respective lag periods of the culture in the agitated skimmilk. When the limits of error in determining the lag phases are considered these differences were insignificant. Skimmilk without sodium thioglycollate, which was agitated at 2-day intervals as previously described, required a prolonged period (22 days) in order to establish favorable conditions for culture development (Table 2). In this skimmilk, not containing sodium thioglycollate, the culture eventually developed. It is theorized that milk contained components which acted much in the same manner as sodium thioglycollate since the skimmilk was eventually reduced to a level consistent with growth (Table 2). The agitation introduced air into the medium which temporarily exhausted the reducing capacity of this naturally occurring oxidation-reduction system. Important factors which affect the autoreductivity of the skimmilk medium are the time periods between agitation, the amount of air introduced during agitation, and the effectiveness of the method of dispersing the air throughout the medium. It is obvious that the 2-day period between the agitations employed in the initial studies (Table 2) delayed but did not inhibit the culture development of C. botulinum. Somewhat longer periods between agitations in the control sample of skimmilk (Table 12) reduced the lag phase of culture

Table 17. Comparison of Lag Phases of Culture Development of C. botulinum Type 62 A Under Varying Conditions

Fatty Acids	Length of Lag Phase			
	Skimmilk		Trypticase	
	Agitated	Unagitated	Agitated	Unagitated
	(days)			
Control	20	15	3	3
Capric	26	23	5	5
Caprylic	24	22	5	6
Myristic	26	23	6	5
Stearic	26	24	6	6

development by approximately 2 days; this difference was considered to be a slight decrease when the lag phase in this control sample is compared to the lag phase determined from the data in Table 2. However, when the skimmilk was not agitated (Table 13) the lag phase was decreased by 7 days.

Trypticase medium containing sodium thioglycollate, as previously described, provided favorable conditions for culture development. Early trials indicated that the culture would not develop in 4% Trypticase solution which did not contain sodium thioglycollate. In the control 4% Trypticase medium containing sodium thioglycollate the lag phases of culture development in the agitated and unagitated samples were the same, 3 days (Table 17). No appreciable differences were observed in the lag phases of the agitated and unagitated samples containing capric, caprylic, myristic, and stearic acids. Marshall (1963) reported that in 22 days of incubation at 18 C, 0.2% sodium thioglycollate in agitated skimmilk sterilized at 121 C for 12 min and which contained approximately 1,000 spores per ml, did not reduce the lag phase of culture development of C. botulinum. In a similar series, skimmilk samples which were not agitated Marshall (1963) showed that sodium thioglycollate reduced the lag phase of development by approximately 14 days.

The ability of the culture to develop more rapidly in Trypticase, under the same conditions of incubation and agitation as the skimmilk medium, can possibly be attributed to the fact that Trypticase, an enzymatic digest of casein, may present a more favorable substrate for microbial attack than does the larger and less soluble protein of milk, casein. Although the oxidation of the sulfhydryl groups of the thioglycollate ions probably occurred during agitation, the Trypticase medium readily became reduced and permitted the rapid development of the culture. The introduction of oxygen into the unagitated samples was limited to that which could diffuse into the Trypticase medium from

the surface. In these samples the sulfhydryl groups were not inactivated by the oxygen as in the agitated samples, and Trypticase medium was readily reduced when sodium thioglycollate was present. The agitated Trypticase medium, containing sodium thioglycollate, was also rapidly reduced and permitted the culture to develop in the same period as the unagitated medium.

The prolonged lag phase of culture development of C. botulinum (Table 2) can be attributed to a number of factors. The inoculum level directly affected the length of the lag phase of culture development. When the inoculum level was low the lag phase was always longer than the lag phase obtained with a larger inoculum. This relationship was true at all incubation temperatures which allowed for culture development of C. botulinum. Another factor which influenced the development of the culture was the presence of fatty acids in the medium. Some fatty acids were instrumental in prolonging the lag phase although the concentration of acids tested exceeded (10-fold increases in some cases) the concentration of these acids reported in fresh milk (Harper et al., 1961). It is important to consider that the mixtures of the acids were effective in completely inhibiting the development of the culture. In a 4% Trypticase medium, which was more suitable for culture development than skimmilk, a fatty acid mixture containing individual fatty acids at a concentration of 0.0001%, extended the lag phase for 3 days. This concentration of each acid was consistent with the concentration of the acids reported in milk by Harper et al. (1961). They reported that caprylic acid was present in fresh milk at a concentration of 0.0023%. Undoubtedly many more fatty acids naturally occur in milk and it would not be inconsistent to ascribe to them some effect on culture development of C. botulinum. The last factor responsible for the prolonged lag phase of culture development was the presence of air introduced into the skimmilk during sampling. When the periods between samplings

were increased the lag phase became somewhat shorter and when air was not introduced by agitation during sampling the lag phase of culture development became appreciably shorter.

Results of the present investigation on the effect of botulinus toxin on tissue culture indicate that the toxin exerted a toxic effect on some tissue cultures. Not all mammalian cells show sensitivity to the toxin. Some of the cells showed a high degree of sensitivity to the toxin, whereas one tissue culture was completely refractive to the toxic action of the bacterial metabolite. In all cases of toxic reaction, displayed by the tissue cultures, the action could be prevented by the addition of specific antitoxin or by heating the toxin before use.

Botulinus toxin is highly specific in its action on the nervous system. The peripheral nerves are most highly affected, but as indicated by this investigation other cells in culture can likewise be affected by the toxin. Since in vivo, the action of the toxin on the peripheral nerves is extremely rapid, the effect on other tissues in the body are masked by the more pronounced effect on the peripheral nerves. Also, Lamanna (1959) stated that tissue cultures of mammalian cells would be unaffected by the toxin. The action on the nervous system is acute and the peripheral nerves would constitute the major site of attack but other tissues may also be affected. Under the conditions of the present investigation it was found that even though an organized cholinergic system is lacking in tissue cultures toxicity of the component cells was evident. Cytotoxicity of non-nervous tissue can occur.

SUMMARY

Clostridium botulinum Type 62 A grew and produced toxin in sterile skimmilk at 13 and 18 C. At 10 C no growth took place in 56 days. C. botulinum Type B grew and produced toxin in skimmilk at 18 C but did not grow at 13, 10 and 7 C after incubation for 56 days. An amino odor was always detected at least 2 days prior to the production of lethal levels of toxin in the kimmilk. Lethal levels of toxin were detected before a visual change occurred.

Caprylic, capric, myristic, and stearic acids, at a concentration of 0.01% in agitated skimmilk extended the lag phase of culture development for approximately 5 days over that obtained in the control. In unagitated skimmilk these acids extended the lag phase of culture development for approximately 8 days. At a total fatty acid concentration of 0.1% or 0.01%, a mixture of the 10 fatty acids inhibited culture development; the culture failed to develop in 40 days.

Under agitated and unagitated conditions caprylic, capric, myristic, and stearic acids, at a concentration of 0.001%, in 4% Trypticase medium, did not markedly prolong the lag phase. A mixture of the 10 fatty acids at a concentration of 0.01% or 0.001%, in the agitated and unagitated Trypticase medium, extended the lag phase for approximately 3 days.

Tissue cell cultures were affected by Type A botulinus toxin. Detroit-6-Parent and Detroit-6-Yale Clone cultures showed cytopathological changes in the presence of the toxin. Mouse L Culture cells were not affected by the toxin.

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