EFFECT OF OXIDATION-REDUCTION POTENTIAL OF MILK ON CLOSTRIDIUM BOTULINUM 62A

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EFFECT OF OXIDATION-REDUCTION POTENTIAL OF MILK ON CLOSTRIDIUM BOTULINUM 62A

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Robert S. Marshall

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INTRODUCTION

The pattern of culture development of Clostridium botulinum 62A in skimmilk was reported by Kaufmann and Brillaud (1961). Further work in this area undertaken to determine the pattern of culture development in skimmilk and whole milk under a variety of experimental conditions gave variable results. The importance of oxidation-reduction potential in the cultivation of anaerobes has been well established.

The ability of whole milk, which had been heated at 115.5 C for 15 min and held at 50 C for 200-300 min, to demonstrate a negative potential has been shown by Higgenbottom and Taylor (1960). Fontana and Colegrande (1962) demonstrated the reducing activity of sterile whole milk and skimmilk held at 25 C for 8 hours. Since Cl. botulinum 62A is an anaerobe, investigations were undertaken to determine the effects of the oxidation-reduction potential of milk at 18 C on culture development during extended periods of storage.

LITERATURE CITED

The effect of the oxidation-reduction potential on the growth of anaerobes has been reported by a number of authors. Knight and Fildes (1930) showed that the positive limit of oxidation-reduction potential required for germination of Cl. tetani was 110 mv at pH 7.0. Aubel et al (1946) using methylene blue for the determination of E_h, demonstrated the limiting potential of Cl. saccharobutyricum and Cl. sporogenes to be 123 mv at pH 7.0. Rabtonova et al (1955) using nutrient meat broth showed that Cl. sporogenes and Cl. putrefaciens produced reducing agents which helped to reduce the medium during the period preceding multiplication. Ando and Inoue (1957) demonstrated the importance of the reducing properties of squid flesh on the development of Cl. botulinum Type E and the activity of the spores in assisting in the reduction of the medium.

Harland et al (1952) have reported that heating milk to 90-96 C for 60-80 sec imparted greater reducing properties than heating to 76.7-96 C for 30 min, due to more rapid production of reducing groups. Higgenbottom and Taylor (1960) have shown that milk heated to 115.5 C for 15 min and held for 200-300 min has a potential of -300 mv. Fontana and

Colegrande (1962) held sterilized whole milk and skimmilk at 25 C for 8 hrs and obtained potentials of -110 mv and 10 mv, respectively. Much of the variation in redox potential reported for milk products is due to the method of determination. The influence of heat-treatment on the reducing activity of milk has been reported by Crowe *et al* (1948). These workers found that the reducing activity increased upon heating at 90 C for 30 min. Sjostrom and Larrson (1949) supported this finding and noted that pasteurization increased the reducing activity of whole milk to a greater extent than skimmilk. A number of reasons have been postulated to explain the reducing activity of milk following heat-treatment. Whitehead (1931) postulated that the oxidizable fat content of whole milk was responsible for the greater reducing capacity. Townley and Gould (1943); Hutton and Patton (1952); and Dill *et al* (1962) have ascribed the increase in reducing activity of milk after heat-treatment to an increase in sulfhydryl activity. Since heat denaturation of protein increases titratable sulfhydryl groups, heat-treatment of milk would denature the β-lactoglobulin portion of the milk serum proteins resulting in increases in sulfdydryl activity (Hutton and Patton, 1952). Harland *et al* (1952) have demonstrated the ascorbic acid content of milk to be correlated with the E_h. The browning of milk upon heating has been shown by Kass and Palmer (1940) to be associated with increased reducing capacity; methylene blue was used as a redox indicator. Saal and Heuklom (1947) using E_h determinations, have shown that the initial oxidation-reduction potential is a function of the dissolved free oxygen, the ascorbic acid content, and lactoflavin content. Greene and Jezeski (1957) have shown that inhibition and stimulation of starter culture metabolism in heated milk were due to the sulfhydryl and sulfide groups which were produced on heating of the milk substrate; no work was undertaken with anaerobes.

MATERIALS AND METHODS

Preparation of spore inoculum and counting procedure. Spores of Clostridium botulinum 62A were heat-shocked for 10 min in a water bath at 100 C and inoculated into trypticase medium for spore production containing 4% Trypticase (BBL), 1 ppm thiamine hydrochloride, 0.1% sodium thioglycolate, 0.1% potassium phosphate-di-basic, 0.05% sodium bi-carbonate and 0.0004% methylene blue chloride (Day, 1960). In all instances media components were prepared on a weight to volume basis. The culture was incubated at 35 C until direct microscopic examination indicated greater than 90% sporulation. The organisms were harvested by centrifugation in a Servall centrifuge at 5000 G for 30 min, washed and resuspended three times in pH 7.2 phosphate buffer (Black, 1960). The spores were stored at 4 C in pH 7.2 phosphate buffer. The stock suspension was always used within 6 days after preparation.

To determine the spore count, a sample was heat-shocked at 100 C for 10 min and cultured in YESB agar medium (Wynne et al, 1955). YESB agar contained 1% yeast extract, 0.1% soluble starch, 0.2% glucose, 1.6% agar and 0.0004% methylene blue chloride. To make counts, 5.0 ml of sodium thioglycolate-buffer solution were aseptically added to 200 ml of melted

YESB agar which was cooled to 47 C. The sodium thioglycolate-buffer solution was prepared as follows: 15 ml of a sterile 13% sodium thioglycolate solution were added to 35 ml of a sterile solution which contained 13% di-basic potassium phosphate and 2.8% sodium bi-carbonate. To make cell counts, the culture was diluted in freshly prepared pH 7.2 phosphate buffer containing 0.1% sodium thioglycolate, and was pipetted into a sterile flat oval test tube; 20 ml of YESB medium were added rapidly. The tubes were incubated at 35C, and the colonies were counted after 24 hours. Counts were performed in duplicate from separate bottles; this was done to increase the reliability of the counts.

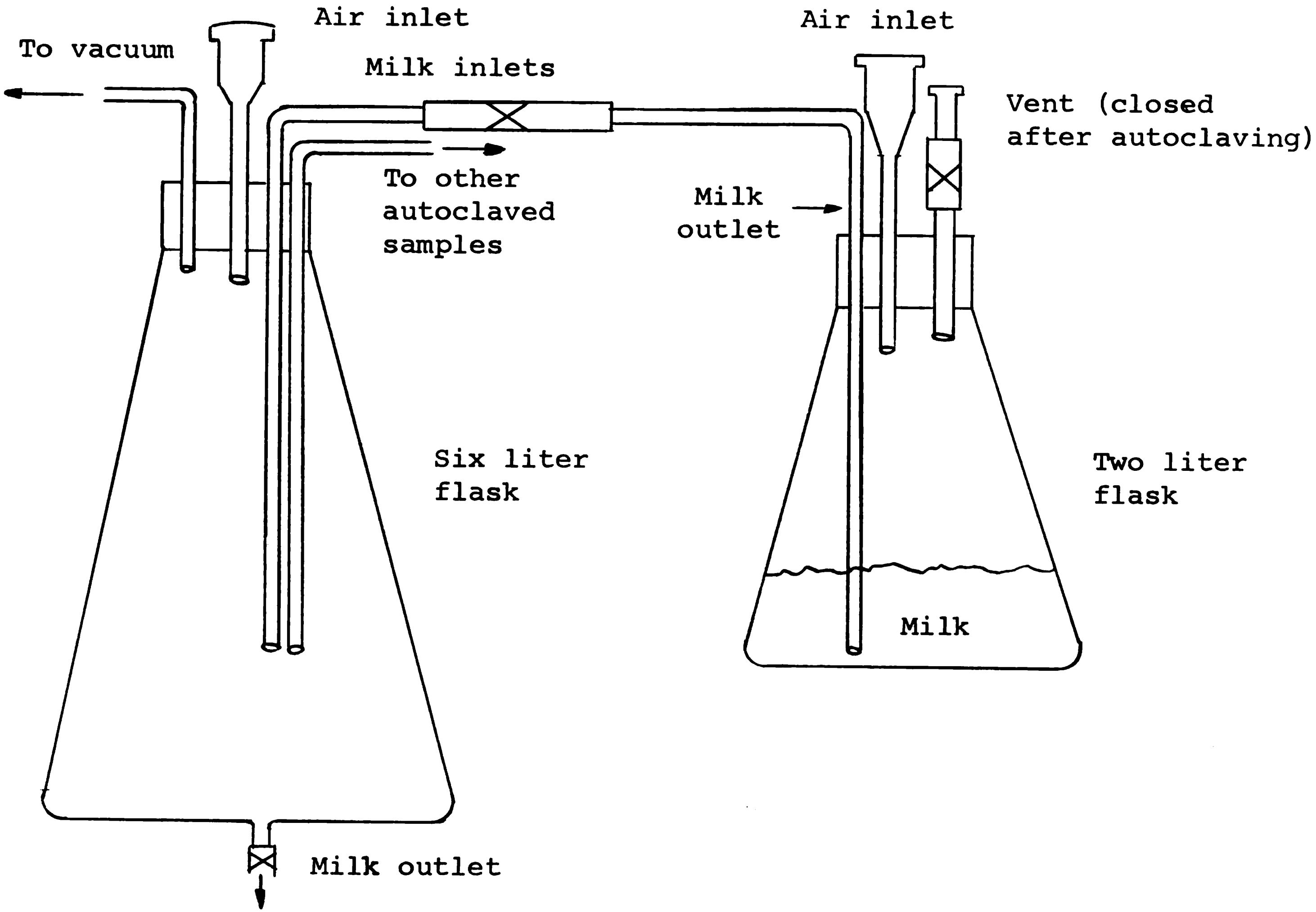
Preparation, dispensation and incubation of media. Milk and trypticase medium were used as culture media in these studies. Raw skimmilk or pasteurized, homogenized, vitamin D fortified, whole milk were obtained from a local dairy. In all instances the individual milk samples were combined before autoclaving to minimize variation. The trypticase medium contained 4% Trypticase, 1 ppm thiamine hydrochloride, and was buffered at pH 6.6 using 200 ml of phosphate buffer solution per liter of medium. The phosphate buffer solution was prepared by diluting a solution of 50 ml of 0.2 M KH_2PO_4

and 17.8 ml of 0.2 M NaOH to 200 ml. The pooling and dispensing apparatus, shown in Figure 1, was required to pool all similar media after autoclaving and to enable large volumnes of media to be dispensed aseptically. All air inlets and vents were covered with four layers of glass wool mat PF 115. The apparatus was wetted and sterilized before use by autoclaving at 121 C for 30 min. The media, sterilized by a variety of methods, were cooled with vigorous shaking in an ice bath at 15 C and transferred to the dispensing apparatus; this minimized variation due to slight unavoidable differences in autoclaving. The pooled milk was aseptically distributed into screw-cap containers. Spores of Cl. botulinum which had been heat-shocked for 10 min at 100 C were used to provide an inoculum of approximately 2×10^3/ml in all trials.

Incubation at 18 C was carried out with and without agitation. One series of samples was inverted four times every two days; these were designated as agitated samples in which the effects of agitation were accumulative. A second series was inverted four times only on the day of testing and discarded after testing; these samples were designated as static.

To detect aerobic contamination, 5 ml of milk were removed prior to making an E_h determination and inoculated into

FIG. 1. Pooling and dispensing apparatus



10 ml of Brain-Heart Infusion broth in a 20 mm x 150 mm test tube. Growth in these tubes after 48 hours at 35 C indicated contamination with aerobic bacteria. Direct microscopic examination was also used to indicate the presence of contamination.

Preparation of electrodes and measurement of E_h. The platinum electrode was made by fusing 25 gauge platinum wire into a soft glass tube 3.0 mm x 150 mm. Three coils were made around an 18 gauge hypodermic needle. Approximately one inch of platinum wire extended into the tube which was filled with filtered mercury to within an inch of the top. Copper wire was immersed into the mercury to establish a junction with the lead wire which was connected to a Beckman H-2 pH meter by means of a Beckman 700-1 terminal adapter. Prior to use the platinum electrode was washed in hot concentrated nitric acid and throughly rinsed in distilled water. The saturated KCl, 2% agar bridge was made from 3.0 mm soft glass tubing. One end of the bridge was 150 mm long to enable the electrode to reach the bottom of the bottle containing the sample; the other end was placed in a saturated KCl solution which contained a standard calomel electrode.

To determine the E_h, 10 ml of sample were aseptically removed from the upper portion of the bottle, and the platinum electrode and agar bridge were lowered to the bottom of the bottle. Care was taken to avoid agitation of the sample. The electrodes remained undisturbed for 10 min in each instance before a reading was made. Rubber gloves were worn during all manipulations to avoid erroneous readings due to grounding of the electrodes. The E_h was determined as follows:

Observed potential in millivolts =
(7.00 -pH scale reading) x 60

E_h = Observed potential + potential of standard
half-cell (246 mv at 22 C)

Since all samples were incubated at 18 C, all E_h determinations were carried out at this temperature; the calomel half-cell however was maintained at 22 C. Readings obtained in the alkaline range indicated a negative potential.

Population curve characteristics. The generation time of Cl. botulinum 62A was calculated from the slope of the line representing the exponential phase of growth. The data was statistically analyzed by the method of least squares to ascertain the slope. The length of the lag phase was determined by the method of Monod (1949).

To detect the presence of toxin, a 0.5 ml sample of medium under investigation was injected intraperitoneally into each of two adult, normal, white mice. Two mice were injected with 0.5 ml of culture medium which had been heated for 10 min at 100 C. The mice were observed for 36 hours; death of the mice which received the unheated material concomitant with the survival of the mice which received the heated material indicated the presence of toxin.

In selected instances 0.5 ml of milk and 0.5 ml of bivalent A and B botulinal antiserum were incubated together in darkness at room temperature for one hour and injected intraperitoneally, in 0.5 ml amounts, into each of two mice. Control mice received milk sample which had been diluted 1:1. Survival of the mice injected with the antiserum-milk mixture, concomitant with the death of the mice which received the milk without antiserum, identified the toxin as being of botulinal origin.

RESULTS

Development of Cl. botulinum 62A in Skimmilk

Raw skimmilk was ditributed in 200-ml volumes into each of twenty 500-ml Erlenmeyer flasks, autoclaved in groups of ten for 10 min at 121 C and cooled to 15 C with vigorous shaking. The contents were pooled and dispensed in 380-ml volumes into each of ten sterile 12-oz prescription bottles. Following inoculation with *Cl. botulinum* 62A, two bottles were incubated as agitated samples and eight bottles were incubated as static samples. The air space in the closed container was approximately 5 cc.

The data, shown in Table 1, indicate that even the mild agitation employed inhibited culture development as no growth occured after incubation for 19 days; in the static sample after a lag phase of 14 days, growth occurred and a level of 8×10^6 cells/ml was attained in 19 days.

Since the mild agitation may have influenced the anaerobic conditions, culture development in skimmilk which contained sodium thioglycolate was investigated using agitated and static samples. One hundred and fifty milliliters of raw skimmilk were placed into each of 40 milk dilution bottles and autoclaved for 12 min at 121 C; the samples were

TABLE 1. Effect of agitation on the growth of <u>Cl</u>. <u>botulinum</u> 62A at 18 C in skimmilk sterilized for 10 min at 121 C

Incubation time	Condition of incubation	
	Agitated	Static
(days)	#/ml x 10^3	
0	3.0	3.0
5	2.2	- -
8	1.7	4.0
11	1.6	4.6
15	1.0	14.0
18	0.65	460.0
19	1.1	8,000.0[a]

[a] Toxin present.

air-cooled, without shaking, to 15 C. Sodium thioglycolate solution was added to each of sixteen bottles to give a final concentration of 0.2%. After inoculation, two bottles with and without sodium thioglycolate were incubated as agitated samples, and the remainder were incubated as static samples. The air space in each container was approximately 10 cc.

As can be seen from Table 2, the presence of sodium thioglycolate in the static sample allowed the culture to develop to a level of 8 x 10^6 cells/ml in 10 days, whereas 22 days were required to reach a level of 2 x 10^6 cells/ml in the absence of sodium thioglycolate. The lag phase was 14 days longer in the latter instance. Even with the addition of sodium thioglycolate the agitation employed inhibited the development of the organisms for the duration of the study.

Development of Cl. botulinum 62A in Whole Milk

Effect of agitation and sterilization time. In view of the fact that whole milk has been reported to be more reductive than skimmilk (Whitehead, 1931; Sjostrom and Larrson, 1949; Larson and Jenness, 1950; Fontana and Colegarande, 1962), and

TABLE 2. Effect of 0.2% sodium thioglycolate in skimmilk, sterilized at 121 C for 12 min, on the growth of *Cl. botulinum* 62A at 18 C

Incubation time	Sodium thioglycolate			
	0.2%		0%	
	Condition of incubation			
	Agitated	Static	Agitated	Static
(days)	#/ml x 10^3			
0	1.7	1.7	1.7	1.7
4	1.4	6.5	3.4	3.3
6	1.6	15.0	2.6	- -
8	1.0	250.0	1.8	2.7
10	1.0	8,000.0	1.5	- -
12	1.0	67,000.0	1.4	2.5
14	0.8	- -	1.2	- -
16	1.0	- -	2.2	1.3
18	1.8	- -	2.2	1.1
19	0.8	- -	1.5	9.0
22	1.6	- -	1.5	2,000.0[a]

[a] Toxin present.

since heat treatment influences the reducing activity of milk (Sjostrom and Larrson, 1949; Larson and Jenness, 1950; Harland *et al*, 1952; Dill *et al*; 1962), it was decided to investigate the effect of the sterilization procedure on the development of the organism in whole milk and to employ E_h as an index of the reducing activity. Whole milk was dispensed in 500-ml volumes into each of sixteen 2-liter Erlenmeyer flasks. Eight flasks of milk were autoclaved for 18 min at 121 C (short sterilization process), and eight were autoclaved for 30 min (long sterilization process). The milk was cooled, with vigorous shaking in an ice bath to 15 C, inoculated, and dispensed in 160-ml volumes into milk dilution bottles. The air space in the closed bottle was approximately 4 cc. Six samples of milk which received the long or the short autoclave treatment were incubated as agitated samples; the remainder were incubated as static samples.

As can be seen from Table 3, the static samples attained cell levels of 3.8×10^6/ml on the 10th day and 3.7×10^6/ml on the 12th day in samples which received the long and short sterilization process respectively. Under the same conditions, the initial E_h values were 192 mv and 234 mv, respectively (Table 4); a decrease of 55 mv was noted in both samples after 3 days. With agitiation the cell levels were 1.4 x

TABLE 3. Effect of agitation of whole milk, sterilized for 18 or 30 min, on the cell count of *Cl. botulinum* 62A

Incubation time at 18 C	Sterilizing time at 121 C			
	18 min		30 min	
	Condition of incubation			
	Agitated	Static	Agitated	Static
(days)	#/ml			
0	4,300	4,300	4,300	4,300
3	440	490	2,000	2,500
5	130	130	710	2,100
8	20	55	1,300	300,000
9	40	22,000	1,400	830,000
10	- -	140,000	14,000	3,800,000
12	- -	3,700,000[a]	210,000	- -
16	12,000	- -	- -	- -
17	340,000	- -	- -	- -

[a] Toxin present.

TABLE 4. Effect of agitation on the E_h[a] of whole milk, sterilized for 18 or 30 min and inoculated with heat-shocked spores of <u>Cl. botulinum</u> 62A

Incubation time at 18 C	Sterilizing time at 121 C			
	18 min		30 min	
	Condition of incubation			
	Agitated	Static	Agitated	Static
(days)	E_h (millivolts)			
0	234	234	192	192
3	203	190	159	144
5	201	177	162	138
8	198	168	159	78[b]
9	192	135[b]	150[b]	-51
10	192	60	93	-154
12	195	-138	-24	- -
17	-3[b]	- -	- -	- -

[a] At pH 6.2 to 6.3.

[b] Increase in cell count detected.

10^4/ml at 10 days and approximately 4 x 10^1/ml at 12 days in the samples which received the long and short sterilization process, respectively. Although the initial E_h values were the same as those obtained with the static samples, the E_h values had decreased by only 30 mv at the end of 3 days.

A lag phase of 5.4 days was observed in milk which received the long sterilization treatment and which was incubated as a static sample; in contrast, the lag phase was 9.0 days in milk which received the short sterilization treatment. In the agitated samples, the lag phases were 9.5 and 14.5 days, respectively. In samples which received the short sterilization process, the decrease in cell count during the lag phase was greater than 99 percent regardless of the conditions of incubation; with the long sterilization process, the decreases were 84 and 51 percent in the agitated and static samples, respectively. It should be noted that agitation inhibited but did not prevent the growth of the organism even after the cell concentration was reduced by 99.9 percent. The decrease in cell concentration may have been due to the dissolved oxygen in the milk or oxidized substances.

Spore germination occurred in all instances. As seen in Table 5, the spore count in the static sample heated for

TABLE 5. Effect of agitation of whole milk, sterilized for 18 or 30 min, on spore counts of Cl. botulinum 62A

Incubation time at 18 C	Sterilizing time at 121 C			
	18 min		30 min	
	Condition of incubation			
	Agitated	Static	Agitated	Static
(days)	#/ml			
0	4,300	4,300	4,300	4,300
3	75	70	320	430
5	25	30	150	270
8	< 10	< 10	70	85

30 min decreased from 4300 to 430/ml in 3 days. It is apparent from the above that approximately 3800 spores germinated; the cell count (Table 3) indicates that 2500 organisms were viable after 3 days. It is possible that the remainder (1300 organisms) died as a result of the products of their metabolic activity in this unfavorable environment. Spore germination occurred to a lesser extent in milk which received the long heat-treatment as evidenced by the fact that 430 organisms/ml remained as ungerminated spores, whereas only 70 organisms/ml remained as ungerminated spores, in the sample which received the short sterilization process. A similar pattern was noted with agitated samples. This might be attributed to heat induced destruction of some factor or the production of toxic products in the samples heated for 30 min.

Effect of holding before inoculation. The variety of biologically active components present in whole milk may be responsible for some or all of the reduction in potential observed during the lag phase. Lepper and Martin (1929) have shown the importance of biological constituents in the reduction of a culture medium. The ability of whole milk to lower the oxidation-reduction potential would provide a favorable environment for the germinated spore; this favorable environment would, to some extent, prevent the loss in cells as evidenced by the decrease in count observed during the lag phase. To investigate this, whole milk was held for a length of time before inoculation. Whole milk was distributed in 500-ml volumes into each of sixteen 2-liter Erlenmeyer flasks, sterilized by both sterilization processes, and dispensed in 160-ml volumes into milk dilution bottles. Three groups of 8 bottles which received the short sterilization procedure were treated as follows: one group was held uninoculated at 18 C as control; one group was inoculated after holding for 3 days, and one group was inoculated on the sixth day. Milk which received the long sterilization process was treated in a similar manner, and was inoculated after 3 and 5 days of holding at 18 C. All groups were incubated as static samples.

In milk sterilized by the short process a holding period of 6 days before inoculation permitted development to a level of 1.3×10^6 cells/ml in 5 days whereas 7 days were required to reach approximately the same level in the sample held for 3 days (Table 6). The results obtained with the milk autoclaved by the long heating process and held for 3 and 5 days reached approximately the same level of cells in 5 and 5.5 days, respectively.

TABLE 6. Effect of holding whole milk, sterilized for 18 or 30 min, on cell count of <u>Cl</u>. <u>botulinum</u> 62A

Incubation time at 18 C	Sterilizing time at 121 C			
	18 min		30 min	
	Holding time at 18 C before inoculation			
	3 da	6 da	3 da	5 da
(days)	#/ml x 10^3			
0	2.5	2.8	2.5	2.1
1	- -	3.3	- -	2.3
2	2.5	2.7	2.3	3.0
3	1.5	76.0	6.0	5.5
4	1.5	560.0	27.0	64.0
5	17.0	1,300.0	240.0	1,200.0
6	720.0	- -	2,500.0	3,500.0[a]
7	1,900.0	- -	3,000.0[a]	- -

[a] Toxin present.

The E_h in the uninoculated control which received the short sterilization process decreased from 231 mv to 216 mv after holding for 3 days; this represents a decrease of 15 mv (Table 7). The sample inoculated after holding for 3 days decreased from 216 mv to 159 mv, a drop of 57 mv, at the same time the uninoculated control decreased from 216 to 186 mv, a drop of 30 mv. The additional drop in E_h (27 mv), was possibly due to the metabolic activity of the inoculum. When the uninoculated sample was held for 6 days the E_h was 186 mv; inoculation at this time reduced the E_h, in 2 days, to a level compatible with growth.

The milk sterilized by the long process was 12 mv lower, initially than that sterilized by the short process; the E_h decreased to a level compatable with growth (156 mv) by the sixth day, in the former; in contrast the E_h in the latter was not suitable for growth after 12 days. Only 2 days were required to establish conditions compatible with growth in milk which received the long sterilization and was held for 3 days, whereas 5 days were required in the sample heated by the short process and held for 3 days before inoculation. Holding the milk, heated by the long procedure, for 5 days before inoculation did not provide more favorable conditions for spore outgrowth than that provided by holding milk, heated by the short process, for 6 days.

TABLE 7. Effect of holding on E_h[a] of whole milk, sterilized for 18 or 30 min, and inoculated with heat-shocked spores of Cl. botulinum 62A

Incubation time at 18 C	Sterilizing time at 121 C					
	18 min			30 min		
	Holding time at 18 C before inoculation					
	Uninoculated	3 da	6 da	Uninoculated	3 da	5 da
(days)	E_h (millivolts)					
0	231	- -	- -	219	- -	- -
1	- -	- -	- -	- -	- -	- -
2	- -	- -	- -	- -	- -	- -
3	216	216	- -	204	204	- -
4	- -	- -	- -	- -	- -	- -
5	187	165	- -	162	135	162
6	186	159	186	156	129[b]	132
7	187	159	174	156	120	120[b]
8	- -	153[b]	153	- -	60	105
9	- -	114	123[b]	- -	-54	33
10	- -	-9	59	- -	-129	-48
11	- -	- -	-30	- -	- -	-138
12	192	- -	- -	156	- -	- -

[a] At pH 6.2 to 6.3.

[b] Increase in cell count detected.

The culture exhibited a lag phase of 1.7 days in the milk which was sterilized for 18 min and which was held for the longest period of time before inoculation. The lag phase was longer by 2 days when the sample was only held for 3 days. Samples heated for 30 min and held for 3 or 5 days prior to inoculation had a lag phase of 2.4 days in the former and 2.1 days in the latter. Regardless of the heat-treatment employed, no decrease in vegetative cell count was observed during the lag phase in samples held for 5 or 6 days before inoculation. In samples which were held for 3 days before inoculation the cell count during the lag phase decreased 40% using the short sterilizing process, and 8% using the long process. The presence of an unfavorable environment for survival is indicated in these cases.

It is apparent from Table 8 that the spores germinated regardless of the sterilizing process or holding period. It is important to note that spore germination occurred at E_h levels higher than that compatable with vegetative cell growth. This compares favorably with the results obtained in earlier studies.

Effect of prolonged heating time. Since previous studies demonstrated that the heat treatment employed during the

TABLE 8. Effect of holding whole milk, sterilized for 18 or 30 min, on spore counts of <u>Cl</u>. <u>botulinum</u> 62A

Incubation time at 18 C	Sterilizing time at 121 C			
	18 min		30 min	
	Holding time at 18 C before inoculation			
	3 da	6 da	3 da	5 da
(days)	#/ml			
0	2,300	2,600	2,500	2,100
1	- -	400	- -	450
2	230	180	700	400
3	90	- -	620	210
4	45	- -	240	- -
5	50	- -	100	- -

sterilization influenced culture development in milk, the effect of prolonged heating on E_h and culture development was investigated. One and one-half liters of whole milk were distributed into each of eight 4-liter Erlenmeyer flasks. The milk was autoclaved, in groups of four flasks, at 121 C for 30 min or 45 min, cooled in ice water, inoculated, and dispensed into milk dilution bottles. It is important to note that the thermal processing received by the milk held for 30 min was less in this instance than that received by the milk in previous studies since the volume of milk in each flask, and the number of flasks autoclaved simultaneously, was greater.

In the agitated sample, which received the prolonged heat treatment, 14 days were required to obtain a cell count of approximately 5×10^5 cells/ml while 16 days were required to reach a level of 860 cells/ml in the agitated sample which was heated for only 30 min (Table 9). In the milk which received the prolonged heat-treatment and was incubated under agitation, 10 days were required to lower the E_h to 153 mv, whereas about 15 days were required to reach this level of E_h in the sample which was heated for only 30 min (Table 10). The lag phases were 11.3 days and 16.5 days respectively.

TABLE 9. Effect of agitation of whole milk, sterilized for 30 or 45 min, on the cell count of *Cl. botulinum* 62A

Incubation time at 18 C	Sterilizing time at 121 C			
	30 min		45 min	
	Condition of incubation			
	Agitated	Static	Agitated	Static
(days)	#/ml x 10^2			
0	19.0	19.0	19.0	19.0
3	2.4	3.1	8.5	12.0
6	1.2	1.2	8.8	16.0
9	.1	1,700.0	8.8	24.0
10	.1	13,000.0	8.3	46.0
11	- -	47,000.0	11.0	1,000.0
12	- -	- -	30.0	6,200.0
14	- -	- -	5,200.0	- -
16	8.6	- -	- -	- -

[a] Toxin present.

TABLE 10. Effect of agitation on E_h[a] of whole milk, sterilized for 30 or 45 min, and inoculated with heat-shocked spores of Cl. botulinum 62A

Incubation time at 18 C	Sterilizing time at 121 C			
	30 min		45 min	
	Condition of incubation			
	Agitated	Static	Agitated	Static
(days)	E_h (millivolts)			
0	257	257	236	236
3	233	215	177	167
6	222	188	171	156
9	205	138[b]	158	134[b]
10	184	90	153	100
11	- -	27	144[b]	78
12	168	-45	138	11
14	162	- -	61	- -
16	150[b]	- -	-12	- -

[a] At pH 6.2 to 6.3.

[b] Increase in cell count detected.

In static samples, the times to reach approximately 1×10^5 cells/ml in the milk heated by the short and long sterilization processes were 9 and 11 days, respectively. The initial E_h of the milk which received the prolonged heat treatment was 236 mv as compared with 257 mv in the sample heated for 30 min. In static samples, the lag phase was 8.7 days when prolonged heat-treatment was employed in contrast with 7.3 days in the sample which received the short treatment. Although the E_h was 21 mv higher in the sample heated by the short time process, the lag phase was 1.4 days shorter than in the sample heated by the prolonged heat-treatment. However, when agitation was employed, the lag phase was 5.2 days longer in the sample which received the short heat-treatment than that obtained in the sample heated by the prolonged heat-treatment.

With the short heat-treatment, the cell count decreased 93% in the static sample and 99.99% in the agitated sample; the cell count decreased 37% in the static sample and 56% in the agitated sample which received the prolonged heat-treatment. It is important to note that in the static samples the cell count decreased 56% more in the sample which received the short heat process than that which received the prolonged heat process. However, the culture developed to a level of

4.7×10^6 cells/ml in 11 days in the former sample, whereas a level of only 1×10^5 cells/ml was reached in the same period of time in the latter sample.

Effect of deoxygenation. The initial E_h of whole milk has been attributed to the dissolved oxygen content (Saal and Heukelom, 1947). The data presented in Tables 10 and 11, show that the E_h of milk decreases on standing. Since the decrease in the E_h of uninoculated milk may be due to the removal of dissolved oxygen by biologically active constituents, it was decided to remove most of the dissolved oxygen by heating to determine the effect of this treatment on E_h and culture development. Four liters of whole milk were autoclaved at 121 C for 18 min, and dispensed while hot in 140-ml or 160-ml volumes into milk dilution bottles. To drive off dissolved oxygen, the bottles were heated to 95 C for 10 min, and 10 ml of hot, sterile mineral oil were added to all bottles containing 140 ml of milk; these were designated as deoxygenated samples. All samples were cooled in an ice bath to 15 C without agitation. The bottles containing 160 ml of milk were shaken 25 times through an arc of 1 ft in 7 sec, to re-oxygenate the samples; these were designated as oxygenated samples. Each bottle was inoculated by immersing a filled, closed, pipette to the bottom of the container.

Uninoculated samples of oxygenated and deoxygenated milk served as controls. All bottles were incubated as static samples.

As shown in Tables 11 and 12, deoxygenation of the milk resulted in an initial E_h of 177 mv which allowed development of the organism to a level of 2.2×10^6 cells/ml in 5 days, whereas in the oxygenated sample the initial E_h was 228 mv, and 8 days were required to reach approximately the same level of cells. The lag phase in the deoxygenated sample was 2.2 days as compared with 5.5 days in the oxygenated sample; the decreases in cell counts during this time were 0% and 98% respectively. Greater than 96% of the spore inoculum germinated in 3 days in both the oxygenated and deoxygenated samples.

Development of Cl. botulinum 62A in Trypticase Medium

In the course of these studies it was observed that milk heated at 121 C for 30 min exhibited more intense discoloration, due to browning, than that which was heated at 121 C for 18 min. It was also observed that culture development proceeded more readily in the former samples than in the latter. Since this type of browning is a result of an

TABLE 11. Effect of deoxygenation on E_h[a] of whole milk, sterilized at 121 C for 18 min, and inoculated with heat-shocked spores of *Cl. botulinum* 62A at 18 C

Incubation time	Oxygenated		Deoxygenated	
	Uninoculated	Inoculated	Uninoculated	Inoculated
(days)	E_h (millivolts)			
0	228	228	177	177
3	219	190	174	159[b]
4	- -	183	174	114
5	- -	174[b]	- -	45
6	192	147	162	-45
7	- -	54	- -	-162
9	183	-114	163	- -

[a] At pH 6.3.

[b] Increase in cell count detected.

TABLE 12. Effect of deoxygenation of whole milk, sterilized at 121 C for 18 min, on the development of <u>Cl</u>. <u>botulinum</u> 62A at 18 C

Incubation time	Oxygenated		Deoxygenated	
	Vegetative cell count	Spore count	Vegetative cell count	Spore count
(days)	#/ml			
0	- -	5,000	- -	5,000
3	630	130	11,000	190
4	130	20	390,000	190
5	1,800	< 10	2,200,000	85
6	21,000	- -	3,500,000[a]	< 10
7	91,000	- -	- -	- -
8	1,100,000	- -	- -	- -

[a] Toxin present.

interaction of lactose with protein and/or protein moities, lactose was added to trypticase medium to give a final concentration of 4%; trypticase medium without lactose was employed as a control. The media were autoclaved at 121 C for 30 min, cooled in an ice bath, inoculated, and dispensed in 160-ml volumes into milk dilution bottles. Uninoculated samples were incubated as controls. Culture development and E_h were followed.

Considerable browning occurred in the lactose-trypticase medium. In the medium containing lactose, the culture attained a level of 5 x 10^6 cells/ml in 5 days, whereas no growth was noted after 17 days in the medium without lactose (Table 13). The initial E_h in the former was 141 mv as compared with 291 mv in the latter (Table 14). In the uninoculated medium without lactose, the E_h dropped slightly (6 mv) in 3 days but underwent no further reduction in the 17 day test period; in the presence of the inoculum, the E_h decreased to 240 mv after 6 days, but never reached an E_h compatible with growth even after incubation for 90 days. Regardless of the high E_h, the spores exhibited greater than 99% germination within 5 days.

In the uninoculated lactose-trypticase medium, the E_h fell slightly from 141 mv to 126 mv in 3 days and increased

TABLE 13. Culture development of *Cl. botulinum* 62A at 18 C in trypticase medium, sterilized for 30 min at 121 C, with or without lactose

Incubation time	Lactose			
	0%		4%	
	Vegetative cell count	Spore count	Vegetative cell count	Spore count
(days)	#/ml			
0	- -	1,8000	- -	1,800
3	1,000	520	1,400	25
5	550	10	5,000,000[b]	< 10
6	540	- -	- -	- -
17	540	< 1	- -	- -

[a] Medium sterilized in the presence of lactose.

[b] Toxin present.

TABLE 14. E_h[a] at 18 C of trypticase medium, sterilized for 30 min at 121 C, with or without lactose[b], and inoculated with heat-shocked spores of <u>Cl</u>. <u>botulinum</u> 62A

Incubation time	Lactose			
	0%		4%	
	Uninoculated	Inoculated	Uninoculated	Inoculated
(days)	E_h (millivolts)			
0	291	291	141	141
3	285	267	126	84
5	285	249	135	-78[c]
6	- -	240	147	-174
17	282	249	216	- -

[a] At pH 6.2 to 6.3.

[b] Medium sterilized in the presence of the lactose.

[c] Increase in cell count detected.

to 216 mv by the 17th day. This pattern of reduction and reoxidation was not observed in any of the studies utilizing milk. Spore counts indicated germination had taken place to the same extent as in the trypticase medium.

DISCUSSION

The ability of sterilized skimmilk to support the growth of Cl. botulinum 62A at 18 C on static incubation has been demonstrated (Tables 1 and 2). The generation time was approximately 10 hrs, and the lag phase of growth was approximately 16 days; toxin was present after about 20 days. Agitation, however, prevented growth. Since agitation of the skimmilk prevented growth and since the lag phase was shortened considerably by the addition of sodium thioglycolate to static samples, it is obvious that the anaerobic state influenced the outgrowth of the culture.

It should be noted (Table 2) that the addition of 0.2% sodium thioglycolate did not bring about a sufficiently reduced state to overcome the effect of the additional oxygen introduced into the system by the mild agitation employed. Almost all of the sulfhydryl groups of the thioglycolate ions may have been oxidized in the presence of this excess oxygen. This can be inferred from the fact that in the agitated sample, no growth occurred in 4 days, whereas sufficiently reduced conditions existed in the static sample to allow development in the same period of time.

In the static samples (Table 2) the quantity of incoming oxygen was limited to that which could diffuse slowly into the skimmilk from the surface; some of the sulfhydryl groups of the sodium thioglycolate were oxidized in reducing this quantity of incoming oxygen, but they were not completely exhausted; a sufficient number remained to maintain the reduced state. The development of the culture in the static sample without sodium thioglycolate indicated the presence of a reducing system in the skimmilk which acted in much the same manner as sodium thioglycolate in that it reduced the medium to a level consistent with growth when the incoming oxygen was limited to that which could be introduced by diffusion. In the absence of sodium thioglycolate, agitation increased the amount of oxygen in the system, thereby overcoming the reducing capacity of the skimmilk.

At pH 6.2-6.3 (the pH of the milk after autoclaving) the limiting E_h for growth was approximately 156 mv, i.e. the organisms did not increase in cell number until this E_h was attained. When corrected for pH by the expression $E_h = E_1 - .06\,\mathrm{pH}$, the limiting potential for <u>Cl</u>. <u>botulinum</u> 62A was 108 mv at pH 7.0. Aubel <u>et</u> <u>al</u> (1946) have demonstrated that the limiting E_h for <u>Cl</u>. <u>saccharobutyricum</u> and <u>Cl</u>. <u>sporogenes</u> was 123 mv at pH 7.0. An E_h of 110 mv at pH

7.0 was found to be limiting for the outgrowth of spores of Cl. tetani by Knight and Fildes (1930).

The fact that a medium has an initial potential in excess of this limiting potential does not preclude culture developement since metabolically active cells may, if present in sufficient numbers, lower the E_h to allow proliferation. The data in Table 4 illustrates this phenomenon with Cl. botulinum 62A in whole milk. In every instance the organisms lowered the unfavorable potential during the lag phase, and growth occurred in all cases (Table 3). Ando and Inoue (1957) and Rabotnova, et al (1955) have also shown that some organisms have the ability to reduce a medium to the degree required for growth. Undoubtedly the metabolic activity of the germinated spores utilized the oxygen in the medium to their detriment. The outcome of such metabolic activity resulted in the death of some of the germinated spores as observed in these studies. The medium, however, was reduced to some extent by this activity and growth eventually occurred.

Whole milk has been shown to be more reductive than skimmilk. It is important to note that the reducing capacity of whole milk was not overwhelmed by the oxygen introduced during agitation, and the medium was reduced to an E_h which was consistent with culture development (Tables 3 and 4).

It is apparent from Tables 4, 9, and 11 that the sterilization process affected the initial E_h of the medium; the E_h was lower in milk which was heated for the longer period of time. This finding agrees with that of Harland <u>et al</u> (1952). The length of the lag phase was shortened in milk having a lower potential. The decrease in the number of cells during the lag phase was also less at the lower initial E_h. In milk which was heated for 45 min, the culture exhibited a longer lag phase than in milk which was heated for 30 min (Table 9). This is undoubtedly due to the occurrence of some toxic factor or the destruction of a nutrient on prolonged heating. Although the E_h was more favorable for outgrowth on prolonged heating the adverse changes induced by the heat-treatment caused a prolongation of the lag phase. When the milk was agitated, the more favorable nutrient conditions which prevailed following the short heat-treatment were unable to compensate for the high E_h brought about by agitation; as a result, the E_h was not compatable with growth at the end of 14 days. In the agitated sample which received the 45 min heat-treatment, The E_h was compatible with growth after 9 days; the maintenance of a lower potential was due to the greater heat-induced reducing capacity. This was more advantageous for outgrowth than the more favorable nutrient environment in the sample which received the short heat-treatment and had a high E_h.

Since holding sterile whole milk before inoculation resulted in a lower E_h value at the time of inoculation, it is evident that milk is auto-reductive (Table 7). It is this auto-reductive property which makes possible the eventual outgrowth of the organisms in milk. The subsequent addition of inoculum lowered the E_h still further. The total reductive activity during the time of the decrease in the E_h was a summation of the effects of the medium and the organisms. Holding the milk medium prior to inoculation allowed the auto-reductive activity of the milk to lower the E_h; as a result, the organisms demonstrated a more rapid response, as measured by the shorter lag phase and the smaller percent decrease in inoculum (Table 6 and Figs 2 and 3); toxin was present when cell levels were in excess of 2×10^6 cells/ml.

The initial E_h values of the milk samples autoclaved for the short and long times differed. Since all conditions of cooling, dispensing and incubation were the same, no difference in the dissolved free oxygen content should exist between samples. Therefore, the difference in the initial E_h must be due, in part, to other chemical constituents.

Lipids have been suggested by Whitehead (1931) as a possible factor affecting the reducing activity. Dill _et al_ (1962) have also shown that volatile sulfhydryl compounds were formed on heating. Kass and Palmer (1940) associated the browning of milk with the reducing capacity while Harland _et al_ (1952) implicated ascorbic acid in this regard.

The removal of oxygen from sterile whole milk by heating at 95 C for 10 min resulted in a decrease in potential similar in magnitude to that observed when whole milk, which received approximately the same heat-treatment, was held uninoculated for about 5 days. It is obvious from the above that the initial E_h reflects both the oxygen content and the reducing groups of the milk. The decrease in potential which is observed on holding is apparently due to the removal of the dissolved oxygen by the reducing components of the milk. The biological activity, i.e. the ability to be auto-reductive, of sterile whole milk is influenced by the amount of heat-treatment employed in the sterilization procedure. This acitivity is responsible to a large extent for the ability of the medium to support the growth of the obligate anaerobe _Cl_. _botulinum_ 62A. Although the germinated spores have the ability to establish a favorable potential they are only able to do so in milk because the medium _per se_ assisted in reducing the system. This is clearly shown in Table 13, where it can be seen that the germinated spores were unable to reduce the potential of the trypticase medium without lactose to a level consistent with outgrowth. In the trypticase-lactose medium, where the reducing intensity was high, the organisms were able to develop; the role of the redox potential of the medium is indicated. Although these studies were carried out using Cl. botulinum 62A, the effect of the auto-reductive properties of a medium on growth should be considered even with organisms other than obligate anaerobes.

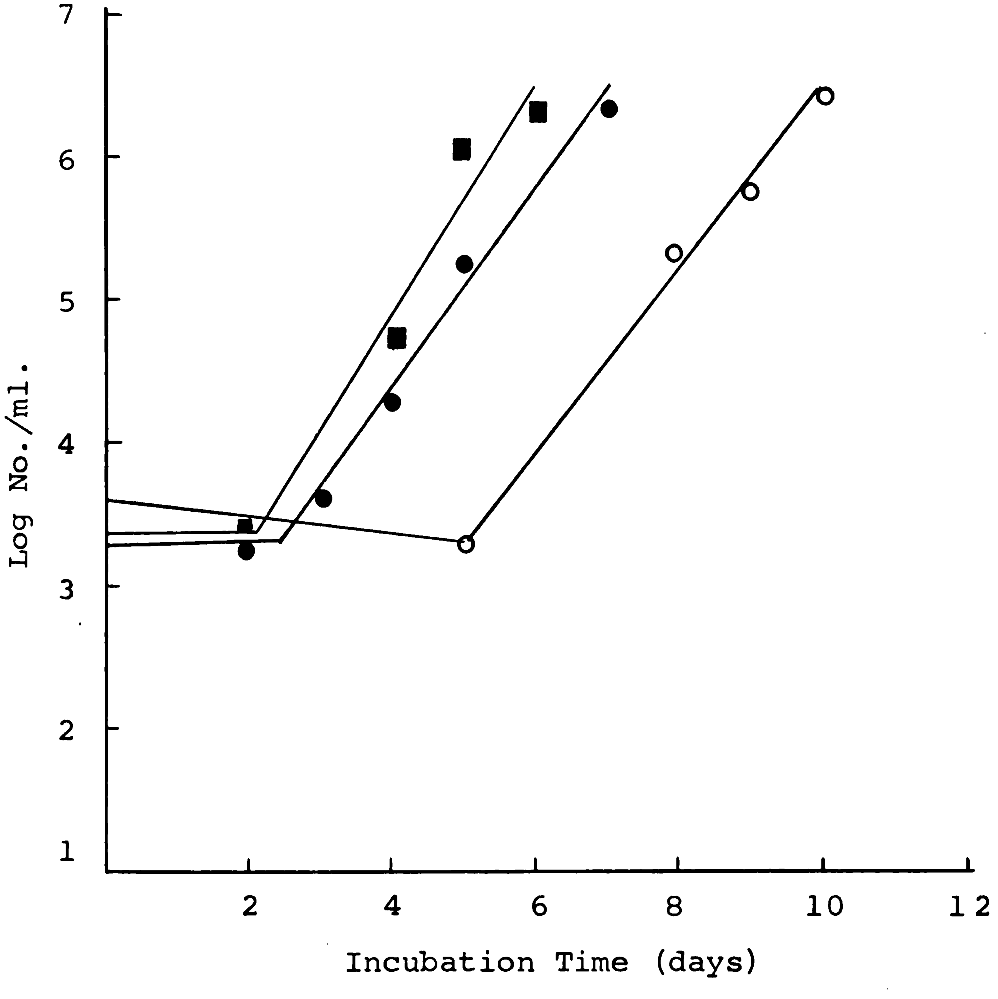


FIG. 2. Effect of holding whole milk at 18 C before inoculation on the development of <u>Cl</u>. <u>botulinum</u> 62A; milk sterilized for 30 min at 121 C (○, no held; ●, held 3 da; ■, held 5 da).

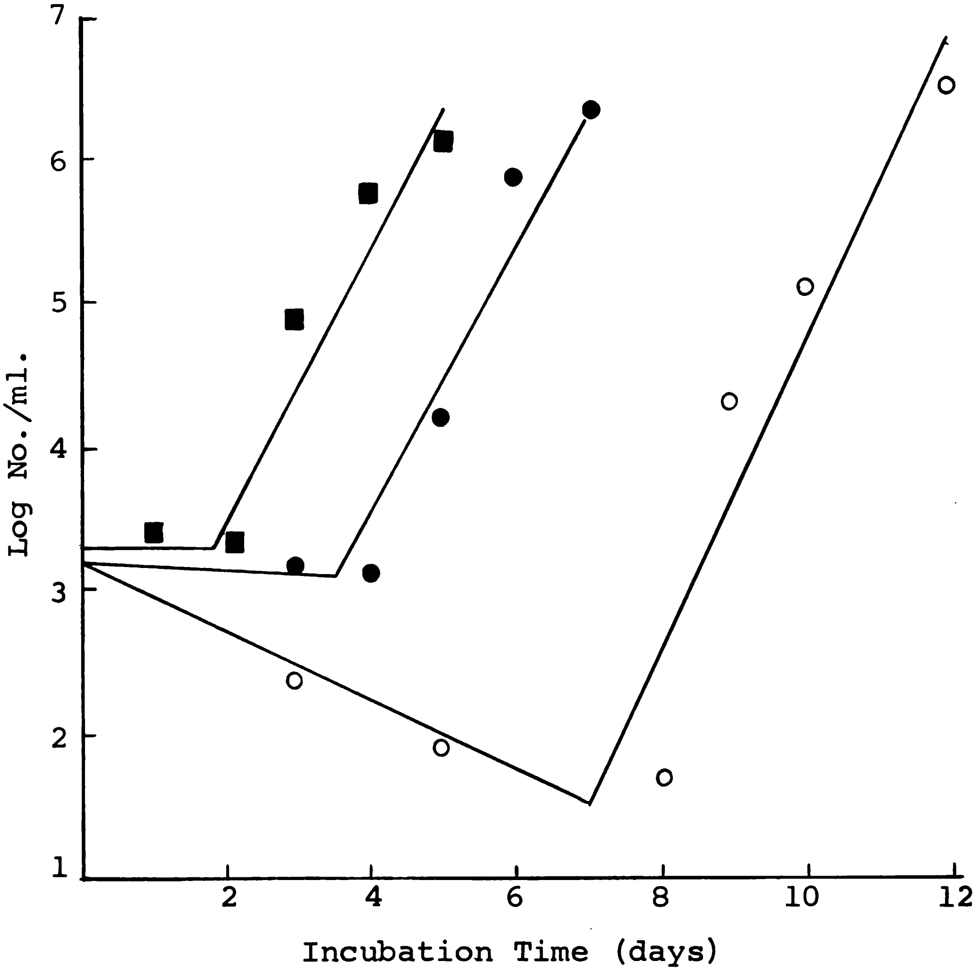


FIG. 3. Effect of holding whole milk at 18 C before inoculation on the development of Cl. botulinum 62A; milk sterilized for 18 min at 121 C (○, not held; ●, held 3 da; ■, held 6 da).

SUMMARY

The developmental response of Clostridium botulinum 62A in milk at 18 C is influenced by the reducing conditions. Occasional, mild, agitation during incubation inhibits growth in skimmilk, sterilized for 12 min at 121 C. Growth occurs when incubation is carried out under static conditions, but the lag phase is 14-18 days. The addition of sodium thioglycolate shortens the lag phase in static samples, but is ineffective in agitated samples.

In whole milk, agitation inhibits but does not prevent culture development. Within limits shorter lag phases result from longer sterilization treatment; prolonged heat-treatment increases the lag phase. Holding sterilized whole milk prior to inoculation shortens the lag phase and decreases the percent loss of cells during this period.

Initial E_h values of whole milk are low in samples which receive a long heat-treatment and high in samples which receive a short heat-treatment. The limiting E_h for development of Cl. botulinum 62A in whole milk is approximately 156 mv at pH 6.2-6.3 (108 mv at pH 7.0). Spore germination, however, occurs at E_h levels higher than this value. Sterile whole milk held uninoculated demonstrated auto-reductive

activity. The addition of inoculum accelerates this decrease in potential. The auto-reductive property of whole milk is responsible, to a large extent, for the development of Cl. botulinum 62A in milk.

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