THE ATTENUATION OF BACTERIA DUE TO TEMPERATURE SHOCK[1]

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Received for publication February 14, 1923

I. INTRODUCTION

The phenomenon of attenuation of bacteria is so closely related to that of stimulation that it seems almost impossible to deal with one without making considerable reference to the other. Within certain limits it may be true that a stimulus for one bacterial faculty, may at the same time attenuate the organism in some other manner before it fails as a stimulant. Heat sometimes alters an organism in such a way that one function may be attenuated while others are not. Pasteur, according to Fisher found that heat may cause the Anthrax organism to lose its virulence while at the same time not losing its ability to grow and to multiply.

Before the discussion of attenuation of bacteria is carried further it is important that the term be defined so as to prevent misunderstanding. The word attenuation is derived from attenuis, to make thin. As used, attenuation means the lessening of one or more functions of bacteria due to abnormal conditions, temporary or prolonged. A wider use of the term was at first considered, that is, the lessening of all of the functions of an organism. However this wide meaning of the term was found untenable due to the fact that our knowledge of bacteria is largely gained by observations of their separate functions. Attenuation is used as an opposite of the term stimulation in the following discussion and

[1] This is a résumé of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in bacteriology in the Graduate School of the University of Illinois, 1922.

refers to one or more bacterial functions and not to the total of bacterial functions. Thus as far as the use of the term goes, a culture of bacteria may be attenuated as to one function and at the same time be stimulated in other respects.

The subject of the attenuation of bacteria divides itself into three important fields of study: (*a*) Attenuation of virulence, a field of science which has revolutionized medical practice; (*b*) attenuation and stimulation of functions of bacteria for commercial advantages, a field of study promising wide application in the manufacturing industries; (*c*) attenuation of the normal, physiological functions of bacteria, phenomena which are rapidly gaining attention because of their relation to the problems of evolution.

There is no intention here to go into the subject of the attenuation of microörganisms as used in producing immunity in living things but simply to mention how thoroughly grounded it is and of how great service to man it has been. Considering the remarkable results obtained in this field it seems rational to believe that other bacterial functions than that of virulence might be manipulated and certain advantages obtained thereby.

There is more or less material to indicate that some of the functions of microörganisms may be altered for definite periods of time. Takamine, in the manufacture of diastase altered *Aspergillus oryzae*. Adams speaks of the modification of yeast cultures in glycerol manufacture. Wehmer reports the loss of oxalic acid producing power by *Aspergillus niger*. Euler and Svanberg speak of temperature adaptation of yeast. LaFevre and Round suggest that certain organisms may acquire salt tolerance and perhaps later require a certain amount of salt for best development. Zikes reports that he changed the generation time of certain yeasts by prolonged cultivation at abnormally low temperatures.

II. PURPOSE OF THIS WORK

It was the general purpose of the following investigation to gather further data as to the possibility of suppressing the bacterial function of multiplication. While it has been generally considered that the increased keeping quality of pasteurized milk

is due entirely to reduction in numbers of bacteria, still the question may be raised whether a part of this keeping quality might not be due to the attenuation of bacteria during the process of pasteurization. It is the object of the following work to measure if possible any change in the generation time of certain bacteria of market milk before and after the temperature shock of pasteurization.

III. METHODS OF STUDY

Thirty-eight samples of raw market milk were plated on one per cent lactose agar and from these plates were selected 144 predominating colonies. These organisms were then added to litmus milk and incubated for fifteen days at 20°C. From litmus milk cultures there were selected at the end of fifteen days, nine representative organisms taking into consideration their action on litmus milk.

Each of the above nine cultures was then taken separately and comparisons made as to the effects of exposure to a temperature of 145°C. for thirty minutes on its rate of multiplication in sterile milk. Table 1 gives the result of comparisons of the generation times of each organism before and after the temperature shock.

To measure properly any possible attenuation which might occur in bacteria subjected to a heat shock, it is necessary to take into consideration certain variable influences in a milk medium which are more or less unfavorable to bacterial development. It has been the belief of some bacteriologists that milk contains antibodies similar to those in blood.

In order to eliminate the many complex factors involved in the use of fresh milk as a medium for the growth of organisms in this study, only separated milk which had been sterilized in the autoclave for one hour at 15 pounds pressure was used. Large amounts of this milk were sterilized at one time so that at least half of the comparisons of generation time of bacteria reported here were made in different small portions of the same batch of milk, all of which was sterilized at one running of the autoclave. These flasks of sterile milk were allowed to stand at 37°C. for several days to make sure of sterility.

TABLE 1

Effect of temperature shock upon bacterial increase

HOURS	UNTREATED BACTERIA PER CUBIC CENTIMETER	INCREASE	HEAT-TREATED BACTERIA PER CUBIC CENTIMETER	INCREASE
Series 8. *B. coli communis* No. 222.1113033 held at 20°C.				
0	1,550		518	
8	7,460	4.8	1,470	2.8
16	55,100	7.3	3,940	2.6
24	337,000	6.1	17,800	4.5
32	2,850,000	8.4	69,000	3.8
40	30,700,000	10.7	230,000	3.3
48	Coagulation		1,090,000	4.7
56			4,100,000	3.7
64			16,600,000	4.0
72			67,000,000	4.0
80			221,000,000	3.2
88			Coagulation	
Series 9. *Bact. lactis acidi* (B) No. 222.2223034 held at 20°C.				
0	26,700		22	
8	280,000	10.4	168	7.6
16	4,190,000	14.9	890	5.2
24	57,100,000	13.6	7,800	8.7
32	586,000,000	10.2	61,000	7.8
40	Coagulation		508,000	8.3
48			3,200,000	6.2
56			32,500,000	10.1
64			257,000,000	7.9
72			Coagulation	
Series 10. *Bact. lactis acidi* (B) No. 222.2223034 held at 20°C.				
0	54,000		1,000	
8	716,000	13.2	7,000	7.1
16	6,740,000	9.4	18,900	2.6
24	61,200,000	9.0	91,000	4.8
32	Coagulation		538,000	5.9
40			3,580,000	6.6
48			29,700,000	8.2
56			182,000,000	6.1
64			659,000,000	3.6
72			Coagulation	
Series 11. Micrococcus No. 222.3333623 held at 20°C.				
0	83		7	
2	98	1.18	6	0
4	212	2.16	11	1.83
6	330	1.55	25	2.27
8	502	1.51	55	2.20
10	940	1.87	82	1.49
12	1,130	1.20	137	1.67

To each of the two flasks of sterile milk in series 9 and 10, there was added 1 cc. of pure culture from a twenty-four hour growth in sterile milk. Then one flask of each series was left raw and one was pasteurized. These two flasks were then brought to the same temperature and agar plates made at the end of various periods as shown in table 1. One per cent lactose agar was used and colonies were counted at the end of two days at 37°C. and three days at 20°C.

In the case of series 8 and 11, the attempt was made to start each flask of a series at as nearly as possible the same number of bacteria per cubic centimeter. This was done by counting the number of bacteria in the milk culture and then calculating how much it would require of the pure milk culture to leave about equal numbers of bacteria per cubic centimeter after pasteurization of one of the duplicates. In order to arrive at this approximate number of bacteria per cubic centimeter, it was found necessary to determine the pasteurization reduction factor for each organism before starting to work with it.

The method of obtaining plate counts was as follows. Five different dilutions of each sample on 1 per cent lactose agar were made and plated in triplicate giving fifteen plates from which to obtain the bacterial count of the sample. In other words the seventeen determinations of numbers of bacteria showing progressive increase of bacteria in series 8 for example were obtained from 255 plates. The fourteen determinations in series 9 were obtained from 210 plates, and the same rule was carried through the determinations given in the other series. It was considered advisable to make what might be called excessive numbers of plates in order that the factor of variation due to the plate method might be reduced to its lowest point. Test tubes containing 9 cc. of sterile distilled water, were used for making dilutions. Vigorous shaking of dilutions was practiced. Care was taken that the 1 cc. of dilution was thoroughly mixed with the agar on each plate. Incubation of plates was for five days at 20°C. and two days at 37°C.

In determining the generation time the method used by Barber has been followed. If the number of bacteria at the beginning of

TABLE 2

Effect of temperature shock on generation times

UNTREATED BACTERIA						HEAT-TREATED BACTERIA					
No. G.	T.	G. T.		Ave. G. T.		No. G.	T.	G. T.		Ave. G. T.	
Data from series 8											
		hrs.	*min.*	*hrs.*	*min.*			*hrs.*	*min.*	*hrs.*	*min.*
2.20	8	3	37	2	54	1.41	8	5	40	4	30
2.84	8	2	48			1.34	8	5	57		
						2.12	8	3	46		
						1.93	8	4	8		
						1.66	8	4	48		
						2.18	8	3	39		
						1.88	8	4	15		
						2.01	8	3	58		
						2.00	8	4			
						1.64	8	4	52		
Data from series 9											
3.31	8	2	24	2	15	2.90	8	2	45	2	47
2.87	8	2	3			2.32	8	3	26		
						3.09	8	2	34		
						2.95	8	2	42		
						3.04	8	2	37		
						2.57	8	3	6		
						3.26	8	2	27		
						2.97	8	2	41		
Data from series 10											
3.65	8	2	11	2	25	2.77	8	2	52	3	36
3.17	8	2	31			1.33	8	6			
3.13	8	2	33			2.20	8	3	37		
						2.47	8	3	13		
						2.66	8	3			
						3.03	8	2	38		
						2.53	8	3	9		
						1.81	8	4	24		
Data from series 11											
0.18	2	11		5	25	0.00	2	0		2	7
1.08	2	1	51			0.83	2	2	8		
0.55	2	3	37			1.13	2	1	45		
0.52	2	3	50			1.10	2	1	48		
0.87	2	2	17			0.49	2	4	4		
0.20	2	10				0.67	2	2	58		

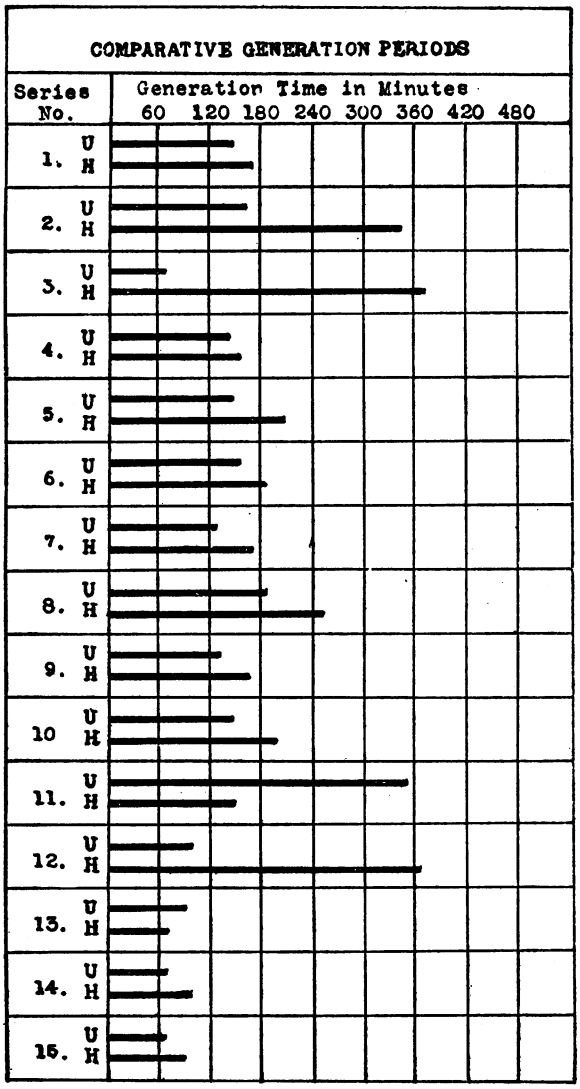
COMPARATIVE GENERATION PERIODS
Series No.
Generation Time in Minutes
60 120 180 240 300 360 420 480
1. U H
2. U H
3. U H
4. U H
5. U H
6. U H
7. U H
8. U H
9. U H
10 U H
11. U H
12. U H
13. U H
14. U H
15. U H

FIG. 1

a period of reproduction (a), and the number at the end of the period (b) be known, then the number of generations (n) can be determined by the formula:

$$2^n = \frac{b}{a}$$

Knowing the number of generations, the generation time can be determined by the following formula:

$$G = \frac{t}{n}$$

The above calculations are based on the assumption that all bacteria in a culure multiply at the same individual rate.

In table 1, the figures under the heading "Increase" were obtained by dividing the number of bacteria per cubic centimeter at the end of an eight hour period by the number at the beginning of the same period of growth. It is only of assistance in aiding the comparisons of rates of increase of bacteria of differently treated cultures. In the graphs showing comparative generation times given in table 1, the letter (U) stands for bacteria untreated by heat and (H) for heat-treated bacteria.

In table 2, "No. G." represents the number of generations which occurred during the period (T) which was eight hours in all the series except 11. The heading (G. T.) stands for generation time in hours and minutes and (Ave. G. T.) represents the average generation time for all the time periods of the series.

DISCUSSION OF RESULTS

Altogether fifteen series of tests were made with fifteen different organisms. Typical results for four of these series have been presented in tables 1 and 2 and the general result of the whole fifteen are graphically presented in the accompanying figure.

In studying the experimental data shown in the graph it is observed that in all the comparative series with the exception of nos. 11 and 13 the average generation time of the bacteria used, has been lengthened by the temperature shock of 145°F. for thirty minutes. Series 11 does not appear to be normal because

of the non-uniformity of the generation time at the beginning and at the end of the growth of the untreated bacteria. It is thought that other factors entered here due to the small number of bacteria per cc., and disturbed the comparisons.

In series 13, 14, and 15 shown on the graph the organisms used were spore bearers and there was no marked difference in the generation times of the untreated and the heat-treated organisms. The heat shock of 145°F. for thirty minutes is considered so far below the temperature exposure which kills the culture that comparisons were of little value in determining attenuation.

In the work with *B. subtilis* spores (series 13) more rapid multiplication was exhibited after than before heat treatment. It would seem that the heat shock of 145°F. for thirty minutes in some way caused stimulation rather than attenuation and it is probable that the temperature exposure is not high enough to threaten in any way the life forces of the spore.

From some of the literature concerning the heat resistance of spores it is evident that this is a very complex subject. H. Weiss reports that the spores of *B. botulinus* are normally destroyed in five hours at 100°C.; forty minutes at 105°C.; and six minutes at 120°C., and that spores one month old are three times as resistant as spores five months old. G. S. Burke found that the spores of *B. botulinus* are more highly resistant to heat when grown in brain cultures than in broth cultures. She reports that exposure to 100°C. or above attenuates the spores to such an extent that the incubation period of the spores is very much lengthened.

It seems very likely that different individual bacteria of a pure culture do not multiply at the same rate although we would expect the average generation time of several large groups of individuals of a pure culture to be quite constant. However, considering that when highly heat resistant bacteria are treated at the pasteurization temperature of 145°F., some individual bacteria die while some individuals are able to withstand even the temperature of boiling water, one would conclude that there is a marked difference between individual bacteria. When a pure culture of milk is treated to a considerable heat shock, the number of bacteria present is always more or less reduced. Those bacteria

which survive differ in some respect from those which die. If this difference were simply one of age of cells it might explain differences in multiplication rates of untreated and heat-treated cultures on the assumption that young and old cells do not multiply at the same rate.

Taking up this subject from another view point, it may be found that there are other characteristics than difference in age of cells which may accidentally accompany ability to survive a heat shock which may have something to do with the generation time of the cell.

To carry further the discussion of possible explanations of the differences in individual bacteria, considering our slight information of the subject would be mere speculation; but there are several additional factors which should not be overlooked. For instance, in two flasks containing pure cultures of the same organism in sterilized milk, when one culture has been subjected to a heat shock and has suffered a reduction in numbers of bacteria per cubic centimeter, the two cultures are no longer comparable. The two cultures differ by the fact that one has considerable numbers of dead cells present while the other culture has none or comparatively few. If we assume that the dead cells are a benefit to the living because of increased vitamine supply, or change in medium, we might conclude that this is a factor tending to shorten the generation time of the surviving bacteria of the culture treated to a heat shock. On the other hand, the rapid cooling of bacteria after a temperature shock is considered by Bushnell to be an attenuating influence in itself. Bushnell says.

> The influence of rapid cooling after heating is a matter of considerable importance in the canning industry. The only effect possible that this procedure could have would be that of shock to the bacterial cell. This might possibly devitalize the cell in such a way that it would be more easily destroyed by subsequent heating, or perhaps perish slowly, or not be able to grow under such rather unfavorable conditions as exist in the sealed container.

If it is a fact that the sudden cooling of heated bacteria causes a shock to the bacterial cells, this is an important factor in studying their rate of growth.

Another phenomenon occasionally referred to in bacteriological literature is the influence which numbers of bacteria inoculated into media have upon the growth of the culture.

It is reported by Churchman that when too few organisms are inoculated into certain media there is no growth, while a greater number of organisms produces growth. On the other hand we know that beyond a certain point, numbers of bacteria or their by-products check growth more or less.

Because of the many complex factors involved in growth, it is impossible to say definitely that attenuation is the cause of the different rates of growth of bacteria, heat-treated, and untreated.

VI. CONCLUSIONS

1. Assuming that the 15 organisms here used are representative of the flora of market milk and that these organisms would not relatively act differently in raw milk than in the sterilized milk here used, one seems warranted in drawing the conclusion that the lengthining of the period between production and souring of pasteurized market milk is not entirely due to the reduction in numbers of bacteria but is to some extent due to attenuation of bacteria during pasteurization.

2. Contamination of market milk after pasteurization is from the standpoint of keeping quality more important than the same amount and kind of contamination having survived the process of pasteurization.

3. In pasteurized milk some species of bacteria are killed outright, some are attenuated, and some are stimulated, depending on the resistance of the organism to heat. Those organisms which have a thermal death point below the pasteurizing temperature and time are of course killed off. Those organisms here tested which have a thermal death point a little above the temperature and time of pasteurization are attenuated markedly, while those which have a thermal death point far above the pasteurization time and temperature are attenuated very little or are stimulated to more rapid growth at least for a period.

4. In the development of methods of increasing the commercial life of some preserved food products, it seems very likely that

some benefit can be obtained by emphasizing the attenuation of microörganisms along with their partial destruction.

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