ADAPTATION OF MASTITIS STREPTOCOCCI TO MILK

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It is well known that fresh milk or milk heated at 58° or 60°C. for 20 minutes will inhibit the growth of a variety of organisms, while when milk is heated at a temperature of 80°C. or more the inhibitory principle is destroyed. That different streptococci behave differently when introduced into the same milk is brought out by the following observation: The growth of the nonhemolytic mastitis streptococcus is inhibited during the first 6 or 8 hours following inoculation and then growth begins and continues rapidly; but scarlet fever streptococci implanted in portions of the same milk gradually diminish in numbers until the milk finally becomes sterile. Both organisms grow readily in milk that has been boiled for 5 minutes.

It appeared to us that a more careful study of the end of the lag phase and the beginning of growth in the case of the mastitis streptococcus might throw some light on the relation between the inhibiting agent and the implanted organisms and perhaps help to explain the mode of action of the inhibitory substance. With these points in view a series of experiments was undertaken.

Experimental

Milk was obtained principally from two cows. Care was taken to prevent contamination by drawing the milk directly from the cleansed udder into sterile bottles. It was chilled at once, freed of fat by centrifugation, heated at 58°C. for 20 minutes, and refrigerated until used. As a rule it was 2 or more days old when inoculated. The culture usually employed was the nonhemolytic mastitis streptococcus which had been kept on artificial media. Subcultures for inoculation were made in broth and used after incubation at 38°C. for 16 hours. Further details are recorded in the protocols.

The effect of milk on the growth of the mastitis streptococcus is considered in Experiment 1.

Experiment 1. Proportionate quantities of milk heated at 58°C. for 20 minutes or boiled for 5 minutes were inoculated with the diluted broth culture of the mastitis streptococcus and incubated at 38°C. in a water bath. Portions were plated with 12 cc. of melted agar (veal infusion) immediately after inoculation and at intervals thereafter, and the colonies counted after 24 hours incubation at 38°C. Previous

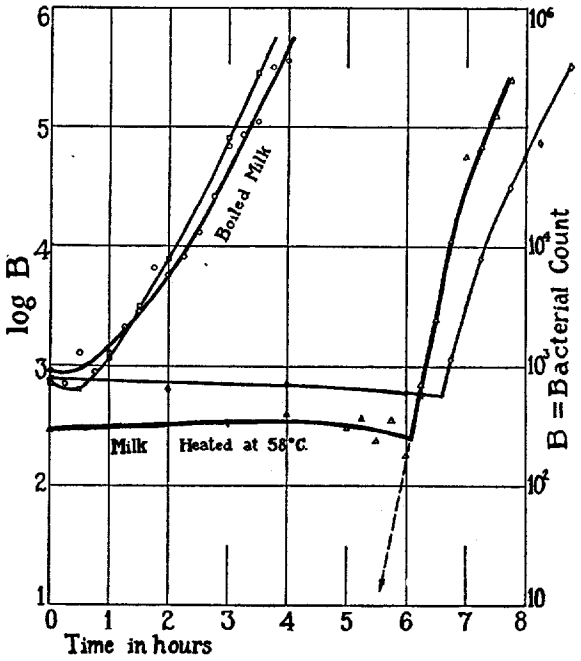

Fig. 1. Retardation of bacterial growth in milk previously heated at 58°C. for 20 minutes as compared with growth in boiled milk.

tests had shown that the lag period was relatively short in the sample which had first been boiled, so that platings were made at 15 minute intervals from the time of inoculation. The milk which had been heated at 58°C. was known to be inhibitory for this particular strain during the first 4 or 5 hours and it was only after 5 hours that frequent platings were made. The results are given in Table I and plotted in Fig. 1. They are typical for this organism under these conditions.

A similar experiment in which the intervals between platings were increased to 30 minutes showed substantially the same results, and the results have also been plotted in Fig. 1.

It will be noted from the data submitted that in the milk heated at 58°C. for 20 minutes the organisms tend to decline slightly in numbers during the first 6 hours. This phase is followed by a sharp "break" in which the organisms begin to multiply at a rapid rate and continue to do so throughout the period of observation. In the boiled milk the lag does not last longer than the first hour after which rapid growth

TABLE I

Growth of Mastitis Streptococcus in Milk

Milk boiled for 5 mins.			Milk heated at 58°C. for 20 mins.		
Time in hours after inoculation	B = bacterial count	Log B	Time in hours after inoculation	B = bacterial count	Log B
0	896	2.95	0	320	2.47
0.25	704	2.85	4.0	412	2.60
.50	1,280	3.11	5.0	307	2.48
.70	896	2.95	5.25	371	2.57
1.00	1,280	3.11	5.50	243	2.38
1.25	2,110	3.32	5.75	345	2.55
1.50	3,780	3.45	6.0	179	2.26
1.75	6,460	3.81	6.25	665	2.85
2.00	5,760	3.76	6.5	2,430	3.38
2.25	8,060	3.91	6.75	11,000	4.04
2.50	13,300	4.12	7.0	57,600	4.76
2.75	26,900	4.42	7.25	69,100	4.84
3.00	69,100	4.84	7.5	127,000	5.10
3.25	85,100	4.93	7.75	250,000	5.40
3.50	108,000	5.04	8.0		
3.75	313,000	5.50			
4.00	350,000	5.55			

begins. Repeated experiments have regularly shown that the onset of growth is sudden in milk heated at 58°C.

Three explanations to account for the sudden onset of growth in the milk previously heated at 58°C. for 20 minutes presented themselves: (1) That the inhibitory substance is utilized at some time during the lag period with the result that the uninhibited organisms grow rapidly; (2) that a resistant form is present from the start but only slowly multiplies to an appreciable number; and (3) that at some time during the lag period a resistant form develops which is capable of rapid

growth. The next three experiments were designed to test the importance of these factors.

TABLE II

Demonstration that the Inhibitory Substance Is Not Utilized during the Lag Phase

(B = bacterial count, *i.e.*, number of colonies per cc.)

A

Time in hours after inoculation	Boiled milk		Milk heated at 58°C.	
	B	log B	B	log B
0	384	2.58	358	2.55
2	4,740	3.68	409	2.61
4	86,000	4.93	499	2.70
5			1,340	3.13
5.5			1,730	3.24
5.75			2,500*	3.40*
6.0			3,700†	3.57†
6.25			9,340¶	3.97¶
6.5			26,600	4.43

B

Behavior of the Mastitis Streptococcus in Portions of A Removed at Various Times

Time in hours	5½ hr. portion,* boiled		6 hr. portion,† 58°C.		6½ hr. portion,¶ 58°C.		Control portion reheated at 58°C. after 6 hrs. incubation	
	B	log B	B	log B	B	log B	B	log B
0	576	2.76	768	2.89	640	2.81	768	2.89
1	2,300	3.36	435	2.64	512	2.71	410	2.61
2	9,120	3.96	420	2.62	576	2.76	435	2.64
3	46,100	4.66	450	2.65	380	2.58	340	2.53
4	∞	∞	250	2.40	1,540	3.19	320	2.50
5	∞	∞	2,000	3.30	32,000	4.50	310	2.49
6	∞	∞	5,760	3.76	∞	∞	510	2.71

* † ¶ The symbols indicate portions of A removed, heated and tested in B.

Experiment 2. Milk handled in the same manner as in Experiment 1 was divided into two lots. One lot, for control purposes, was boiled before inoculation and plated at 2 hour intervals during incubation at 38°C. The other lot was heated at 58°C. for 20 minutes, and likewise inoculated when cool. Portions of it were plated at 2 hour intervals for a time. The intervals were later decreased to

15 minutes. 10 cc. samples were removed after $5\frac{3}{4}$, 6, and $6\frac{1}{4}$ hours incubation. The first tube of inoculated milk, removed after $5\frac{3}{4}$ hours incubation, was boiled 5 minutes; and the other two, removed at 6 and $6\frac{1}{4}$ hours, respectively, were heated at 58°C. for 20 minutes to kill the implanted streptococci. A fourth tube of milk handled in the same manner but uninoculated was incubated for 6 hours and reheated at 58°C. It served as an additional control. All four samples were kept in the refrigerator overnight and in the morning were reinoculated with the culture and incubated at 38°C. Portions were plated each hour. The data are given in Table II and Fig. 2.

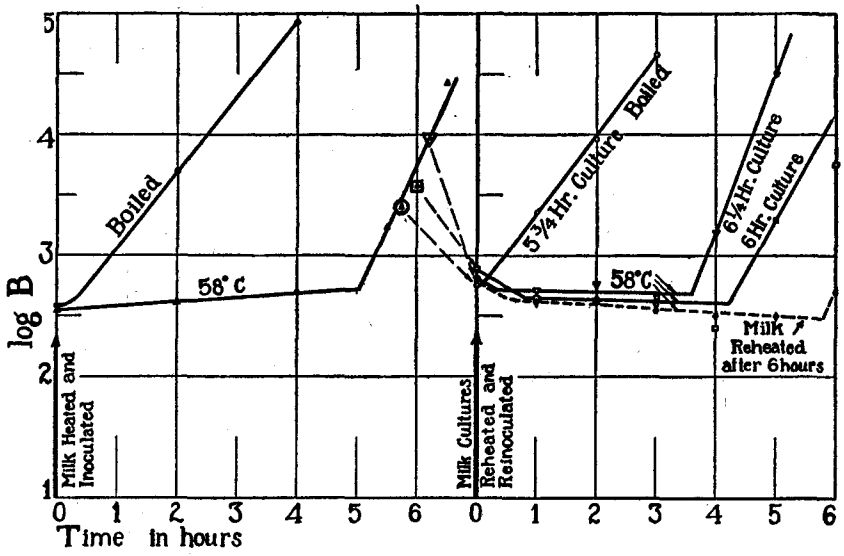

Fig. 2. Demonstration that the inhibitory agent is not utilized during the lag period.

It will be noted that the samples removed from the inoculated milk after 6 and $6\frac{1}{4}$ hours and then heated sufficiently to destroy the inoculated streptococci were still capable of producing considerable lag. It is evident that, though growth had taken place, the principle had been only slightly utilized. The contents of one of the control tubes inoculated and incubated for $5\frac{3}{4}$ hours and then boiled afforded a good culture medium from the first, whereas the action of the principle in the uninoculated milk had not been impaired either by incubation or repasteurization.

Since this experiment showed that the inhibitory substance had not been completely utilized, attention was turned to the possibility of an adaptation to the inhibitory substance on the part of the organism. The next two experiments furnished evidence compatible with the view that the organism adapts itself to the inhibitory principle.

TABLE III

A. *Effect of Fresh Milk on the Mastitis Streptococcus during the Lag Phase and the Beginning of the Growth Phase*

Time in hours	Boiled milk		58°C. milk	
	B	log B	B	log B
0	512	2.71	640	2.80
2	3,840	3.58	434	2.64
4	39,000	4.59	512	2.71
4.5	87,700	4.94	578*	2.76
5.5	495,000	5.70	1,540†	3.19
6.5	∞	∞	13,100¶	4.12
7.5	∞	∞	67,000§	4.83

B. *Subcultures Made by Centrifuging A and Exposing the Streptococci to the Action of Fresh Milk (Heated at 58°C.)*

Time in hours	4½ hr. culture*		5½ hr. culture†		6½ hr. culture¶		7½ hr. culture§	
	B	log B	B	log B	B	log B	B	log B
0	1,020	3.01	2,050	3.31	14,100	4.15	65,300	4.81
0.5	1,020	3.01	3,580	3.55	20,500	4.31	76,800	4.88
1	1,020	3.01	5,450	3.74	28,800	4.46	107,000	5.03
2	7,040	3.85	26,800	4.43	78,000	4.89	214,000	5.33
3	14,100	4.15	47,400	4.68	212,000	5.33	506,000	5.70
4	58,900	4.77	494,000	5.69	730,000	5.86	∞	∞

* † ¶ § These symbols indicate subcultures in B derived from the respective portions of A.

Experiment 3. 25 cc. of fresh milk which had been heated at 58°C. for 20 minutes, and 5 cc. of the same lot which had been boiled for 5 minutes were inoculated with proportionate quantities of mastitis streptococci. Both tubes were incubated at 38°C. and portions plated at regular intervals. 5 cc. portions of the milk that had been heated at 58°C. were removed at 4½, 5½, 6½, and 7½ hours and centrifuged. The streptococci obtained from the sediment were mixed with

5 cc. of fresh milk previously heated at 58°C. and again incubated. Care was taken not to chill the tubes during the process and the fresh milk was warmed to 38°C. before it was added. Portions of each series were plated at intervals. The results are given in Table III and Fig. 3.

It will be seen from the data that the period of inhibition extended to about the 5th hour in the milk that had been first heated at 58°C.,

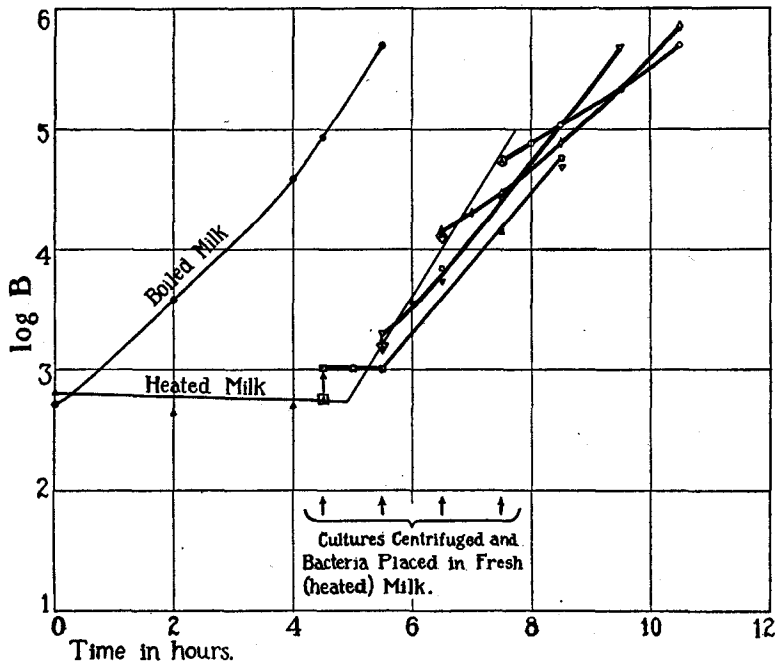

Fig. 3. Effect of fresh (pasteurized) milk on the rate of growth of mastitis streptococci removed from the original milk culture.

then the usual sharp break occurred and rapid growth ensued. The bacteria removed about one half hour before the lag phase ended failed to multiply during the first hour of exposure in fresh milk but grew rapidly thereafter. Thus in spite of the exposure to fresh milk the period of lag was about the same. On the other hand the organisms removed during the active growth phase ($5\frac{1}{2}$, $6\frac{1}{2}$, and $7\frac{1}{2}$ hours after inoculation) all grew without lag in the fresh milk.

Experiment 4 shows that the streptococcus adapts itself to milk.

Experiment 4. Milk previously heated at 58°C. for 20 minutes was distributed in sterile tubes as follows: 1 tube containing 5 cc., and 4 tubes each containing 4 cc. To the tube containing 5 cc., 0.1 cc. of a 16 hour broth culture of the mastititis streptococcus was added and a sample plated at once to determine the number of streptococci. The tube was then placed in the water bath at 38°C. At the end of 1½ hours, 1 cc. was removed and added to one of the tubes containing 4 cc. of milk. A portion of the contents of the original tube was plated as was the

TABLE IV

Total time of incubation in hours	Tube No.	Contents	Time of incubation for each tube in hours	Colonies in plates $= \frac{BD}{625}$	Log $\frac{BD}{625}$	B = calculated bacteria per cc.
		A. *Adaptation of the Mastitis Streptococcus to Frequently Added Milk*				
0	1	0.1 cc. culture + 5 cc. milk	0	1,600	3.20	1,000,000
1½			1½	1,280	3.11	800,000
1½	2	1 cc. from Tube 1 + 4 cc. milk	0	1,088	3.04	136,000
3.0			1½	832	2.92	104,000
3.0	3	1 cc. from Tube 2 + 4 cc. milk	0	896	2.95	22,400
4½			1½	704	2.85	17,600
4½	4	1 cc. from Tube 3 + 4 cc. milk	0	768	2.87	3,840
6.0			1½	665	2.82	3,330
6.0	5	1 cc. from Tube 4 + 4 cc. milk	0	640	2.81	640
7½			1½	3,456	3.54	3,456
		B. *Control Experiment with Boiled Milk*				
0	1	0.1 cc. culture + 5 cc. boiled milk	0	1,280	3.11	800,000
1½			1½	10,300	4.01	6,440,000
1½	2	1 cc. from Tube 1 + 4 cc. boiled milk	0	11,300	4.05	1,410,000
3.0			1½	52,400	4.72	6,550,000
3.0	3	1 cc. from Tube 2 + 4 cc. boiled milk	0	57,000	4.76	1,430,000
4½			1½	∞	∞	∞

mixture in the second tube. After incubation for another 1½ hours, 1 cc. of the second tube was added to 4 cc. of milk, and plates were prepared as before. This procedure was repeated at the end of 4½ hours and 6 hours, so that the inoculated streptococci were regularly diluted and exposed to the action of fresh milk at intervals of 1½ hours. A control series was carried on in boiled milk. The plate cultures were always prepared with the same amount of fluid.

With respect to the first tube the five tubes had dilutions of 0, 1:5, 1:25, 1:125, and 1:625 respectively. In order that the bacterial counts might be comparable the milk was diluted with sterile NaCl solution before plating, as

follows: 1:625, 1:125, 1:25, 1:5, and 0, respectively, thus making all platings on a dilution of 1:625 of the mixture in the first tube.

If B is the bacterial count per cc. and D is the dilution (1, 5, 25, etc.), then BD/625 represents the count in each plate. These values are given in Table IV.

In spite of the fact that the mastitis streptococcus was exposed to the action of fresh milk at intervals of $1\frac{1}{2}$ hours it was able to establish

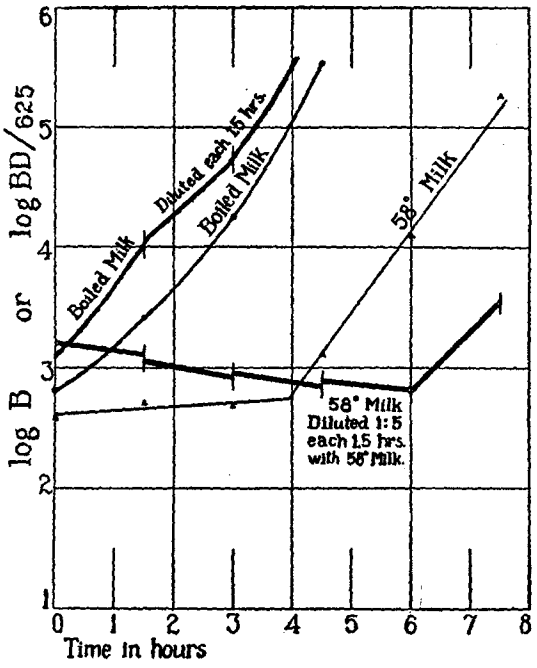

Fig. 4. Effect of milk added at frequent intervals on the growth of the mastitis streptococcus. The bacterial counts are corrected for the amount of dilution.

itself and begin to grow. The procedure, however, had two recognizable effects: first, it increased the lag period from about 4 hours in the milk control tube to about 6 hours in the experimental series, and further, there was a gradual but consistent decline in the number of streptococci until growth began. When the same experiment was done with boiled milk the streptococci increased throughout and the addition of fresh portions of boiled milk failed to retard growth.

Aside from the fact that the mastitis streptococcus becomes adapted to the inhibitory effect of milk the results in Experiment 2 indicated that a small portion of the principle had been utilized during the early phase of active growth. Experiments earlier reported[1] have shown that when milk is heavily seeded with bacteria and incubated sufficiently long to insure multiplication the inhibitory effect disappears. The question arises as to whether the utilization can be considered an adsorption by the bacterial cells. Experiment 5 is designed to throw light on this phase of the problem.

TABLE V

Bactericidal Activity of Milk Previously Treated with Living or Dead Scarlet Fever Streptococci

Time in hours	Milk heated at 58°C.		Milk incubated and refrigerated, then heated at 58°C.		Boiled milk (combined from all tubes)
	Incubated with dead streptococci, then centrifuged	Control milk	Incubated with live streptococci, centrifuged and heated at 58°C.	Control milk	
0	428	428	384	384	576
2	345	166	320	217	13,820
4	192	153	230	77	∞
6	12	4	12	2	∞
8	5	1	3	6	∞
24	0	0	0	0	
48	0	0	0	0	

Experiment 5. Mixed milk from five cows was chilled and freed from fat and 15 cc. amounts placed in four sterile tubes. Two of the tubes were heated at 58°C. for 20 minutes. One tube served as control, and to the other were added scarlet fever streptococci from 500 cc. of 48 hour broth culture which had been heated at 60°C. for 20 minutes. The two other tubes were not heated until after incubation. To one of these the scarlet fever streptococci from 500 cc. of 48 hour broth culture were added. All four tubes were incubated 2 hours and refrigerated 18 hours. The second pair of tubes, which had not been heated, were then heated at 58°C. for 20 minutes. All four tubes were centrifuged and the supernatant fluid was distributed into small tubes and reinoculated with scarlet fever streptococci. The results are given in Table V.

[1] Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1927, **45**, 319.

The experiment was also performed with large quantities of mastitis streptococci. In no case was the inhibitory substance appreciably removed by dead streptococci or by viable organisms, provided always that the tubes were not incubated sufficiently long to permit growth.

DISCUSSION

The experiments have shown that when mastitis streptococci are introduced in small numbers into milk previously heated at 58°C. for 20 minutes there is a lag in growth of 5 or 6 hours succeeded by an abrupt increase which continues at a rapid rate (Fig. 1). The experiments further indicate that the inhibitory principle is not destroyed during this period since sufficient remains to inhibit the growth of a similar culture for about 4 hours (Fig. 2). Furthermore had the substance been completely destroyed during the lag period the break in the curve would not have been as sharp.

The explanation that a resistant form is present from the start and requires a period of time to multiply to an appreciable number, can be immediately discarded from the shape of the curves in Fig. 1. Here we have plotted the logarithms of the bacterial count against time of incubation. Milk previously heated at 58°C. is shown to produce a long lag, followed by a sharp break and rapid growth. The experiment was performed enough times and at intervals sufficiently close for us to be certain of the abruptness of the change. Even if only one bacterium of a strain resistant to the inhibitory agent had been present at the time of inoculation, this one bacterium, if it multiplied at the rate shown to be typical of the surviving forms, could have produced a lag of not more than 3 hours. This calculation takes into account the normal lag of 1 hour which is found when the streptococcus is grown in boiled milk. Thus, if we take the curve after the lag period and extrapolate it to zero time, as indicated by the dash line in Fig. 1, we find that if the surviving bacteria were grown from a single resistant organism this organism was not produced until the 5th hour.

The findings justify us in concluding that mastitis streptococci become adapted to the inhibitory agent. Further proof of this conclusion is given by Experiment 3 in which bacteria surviving the lag period are found to grow rapidly, without lag, when placed in fresh

milk. From Fig. 3 it will be seen that mastitis streptococci removed from milk cultures after various periods of incubation and placed in fresh milk behave much as if they had been left in the original culture. The $4\frac{1}{2}$ hour sample had not reached the period of rapid growth and showed a short lag in fresh milk. The streptococci removed after rapid growth showed no lag.

The results of the experiment cited raise the question as to what would occur if the bacteria were frequently placed in fresh milk during the period of incubation. This was tested in Experiment 4, the conditions of which resemble in some respects those found in the udder. In this experiment a milk culture of mastitis streptococci was diluted 1:5 with fresh (pasteurized) milk each $1\frac{1}{2}$ hours. The tabulated bacterial count (B) is corrected for the dilution (D) so that the numerical value of BD/625 represents the bacteria per cc. for all cultures, on the basis of the dilution in the last culture. Fig. 4 shows that in spite of changing the milk each $1\frac{1}{2}$ hours, the streptococcus develops a resistant type in 6 hours, whereas a lag of 4 hours occurred in the control tube the milk of which was not changed. As a result of the repeated additions of milk there was approximately a 25 per cent mortality among the streptococci. When the experiment was performed with boiled milk as a medium, rapid multiplication took place in spite of the frequent dilutions.

It has been shown[2] that scarlet fever streptococci die in milk. Their numbers gradually decline during the first 24 hours and finally the milk becomes sterile. The fact that the effect is not one of lysis is readily determined by microscopic examination, since intact streptococci may be demonstrated in the milk. It would seem that the action of milk against both the bovine and the scarlet fever streptococcus involves a factor which prevents multiplication. In the case of the mastitis streptococcus cell division is prevented for a time but a resistant type that is capable of multiplication develops. The scarlet fever streptococcus is unable to produce resistant forms and hence perishes.

Experiment 5, in which we attempted to adsorb the substance with large numbers of dead or living streptococci, indicates that

[2] Jones, F. S., *J. Exp. Med.*, 1928, **47**, 965.

there is no specific union between bacterial cell and the substance, since the inhibitory character of the milk was not diminished by such treatment. It seems probable that the substance is one preventing multiplication of bacterial cells and is not notably toxic in itself.

SUMMARY

The data here presented indicate that the inhibitory principle affecting the growth of streptococci in milk is not greatly utilized during the lag phase and that the abrupt termination of lag is not due to the utilization of the principle. They further indicate that the sudden beginning of growth cannot be ascribed to a resistant type of streptococcus present in the culture from the first, but to an adaptation occurring during the lag phase. The addition of large numbers of dead or living streptococci to milk, for limited periods, fails to diminish appreciably the inhibitory principle.