UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

III. The Influence of Milk on the Growth of Scarlet Fever Streptococci.

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PLATE 38.

(Received for publication, March 9, 1928.)

The view usually held covering milk-borne epidemics of scarlet fever is that the streptococci originate in the throats of the milk handlers and through contamination gain access to the supply. It is difficult to explain on such grounds the heavy incidence of infection among the consumers of pooled supplies since relatively few organisms would gain access to limited amounts of milk, and when this was mixed with other milk the dilution would be so great that the probable incidence of human infection would be relatively small. It might be argued that the contaminating streptococcus would multiply rapidly in the milk provided the temperature was favorable and that the product reaching the consumer would thus contain large numbers of the streptococci. But to explain severe outbreaks of scarlet fever through milk contamination one would have to assume that the organism gained access in goodly numbers and multiplied rapidly. However certain experiments here to be reported indicate that streptococci of the scarlet fever type are acted upon adversely by milk.

During the observations in connection with the artificial inoculation of Cow 1462 with a streptococcus of the human scarlet fever type a peculiar phenomenon was observed. It was customary to plate the milk from the involved quarters after logarithmic dilutions in salt solution, the first plate of a series containing a 10^{-1} dilution, the second a 10^{-2}, until to the final plate milk diluted 10^{-6} was added.

Toward the end of the observation, when the numbers of streptococci per cc. were not excessive, it was noted that the first plate culture contained streptococci in smaller numbers than one would expect from the number found in the higher dilutions. Furthermore the colonies were very small with correspondingly small hemolytic zones; yet when such colonies were subcultured and replated colonies of the usual size with well defined hemolytic zones developed. Two explanations suggested themselves, first, that the colonies were too numerous in the plate, and second, that an inhibitory substance had developed in the quarter which was active in the 10^{-1} dilution but not in the 10^{-2} dilution. Further observations indicated that overcrowding was not responsible for the change in the character of the colonies. The inhibition appeared to be a property of the milk and as such worthy of more careful study.

EXPERIMENTAL.

The cultures employed have been described in the preceding papers. They were carried for stock purposes on agar slants to which a few drops of defibrinated horse blood had been added. For the experiments transfers were made into veal infusion broth and incubated 16 hours. Inasmuch as the rest of the procedure varied, some details of the individual experiments are given separately.

Experiment 1.—Milk from individual quarters of Cow 1462 was drawn directly into sterile bottles and after chilling freed of fat by centrifuging. That from the quarters artificially infected with the hemolytic streptococcus was combined and portions of this milk and of that from an uninfected quarter were heated at 60°C. for 20 minutes, while other portions were not heated. In addition the cow was bled on the day before the experiment and the serum collected.

The inhibitory activities of the milk and serum were then tested in the following manner. Serum or milk was added in amounts of 0.5, 0.25, and 0.1 cc. to Petri dishes containing 0.5 cc. of defibrinated horse blood. A loop of dilute broth culture of the streptococcus obtained from Cow 1462 was added to 10 or 12 cc. of melted agar cooled to 48°C. and the whole plated. The plates were incubated for 48 hours at 38°C.

Inasmuch as the unheated milk contained many native organisms giving rise to many colonies in the plates, the results were imperfect and for this reason they will not be considered in detail, although it was clear that even under such conditions the growth of the streptococcus was inhibited.

The results of the first experiment are given in Table I.

The fact seems established by the experiment that when milk from either the infected or normal quarters was added to plate cultures containing small numbers of hemolytic streptococci a distinct inhibition resulted. The colonies were smaller and their hemolytic activity greatly diminished or even entirely suppressed, as when 0.5 cc. of milk from the two involved quarters was incorporated in the culture. In the series in which blood serum was mixed and incubated

TABLE I.

The Influence of Blood Serum and Milk on the Size of Colonies and Hemolytic Activities of the Streptococcus from Cow 1462.

	Plate containing	
	cc.	
Blood serum	0.5	30 colonies varying from 1.5 to 1.8 mm. Hemolytic zones 4 mm.
	0.25	20 colonies average 1.8 mm. Hemolytic zones 4 mm.
Milk heated 60°C., from uninfected quarter (R. F.)	0.5	No growth
	0.25	6 colonies averaging 0.4 mm. Indistinct hemolytic zones 0.8 mm.
	0.1	5 colonies 0.4 to 0.6 mm. Hemolytic zones 1.25 to 1.75 mm.
Milk heated 60°C., from involved quarters, R. H. and L. H.	0.5	No growth in 24 hrs. 48 hrs., 5 tiny, non-hemolytic colonies
	0.25	30 colonies 0.25 mm. Hemolytic zones 0.6 mm.
	0.1	6 colonies 0.025 mm. Hemolytic zones 0.75 mm.

with the culture material there was no inhibition. One may infer that the inhibiting property was not derived from the blood.

It seemed possible that the organism employed might be unique in sensitiveness to the inhibitory action of milk. Furthermore the incorporation of as much as 0.5 cc. of milk to the blood agar plate might perhaps alter the nutritive character of the medium and thus prevent normal growth. The more elaborate procedure employed in Experiment 2 was devised to control these features.

TABLE II.

The Effect of Milk on the Character of the Growth of the Scarlet Fever Streptococcus.

Culture	Milk	Plate containing	
		cc.	
F. C.	R. F., filtered	0.5	No growth 24 hrs. 48 hrs., 10 colonies, tiny, surrounded by faint hemolysis
		0.25	384 colonies, average 0.4 mm. Hemolytic zones 0.75 mm., indistinct
		0.1	345 colonies, average 0.6 mm. Hemolytic zones 1 to 1.25 mm., clear
	R. F., heated 58°C. 20 min.	0.5	320 colonies less than 0.25 mm. Hemolytic zones barely perceptible
		0.25	384 colonies average 0.25 mm. Hemolytic zones 0.5 mm.
		0.1	350 colonies, average 0.6 mm. Hemolytic zones 1 mm.
	R. H., filtered	0.5	325 colonies barely visible × 9. Non-hemolytic
		0.25	400 " " " × 9. "
		0.1	384 colonies, average 0.9 mm. Hemolytic zone 3 mm.
	R. H., boiled 5 min.	0.5	428 colonies, average 0.9 mm. Hemolytic zone 3 mm.
	None		384 colonies, average 0.8 mm. Hemolytic zone 3 mm.
Scarlet Fever 55	R. F., filtered	0.5	90 colonies visible × 9. Non-hemolytic
		0.25	576 colonies, less than 0.25 mm. Hemolytic zones 0.5 mm.
		0.1	580 colonies, average 0.5 mm. Hemolytic zones 2 mm.
	R. F., heated 58°C. 20 min.	0.5	No growth
		0.25	640 colonies less than 0.25 mm. Hazy hemolytic zones 0.5 mm.
		0.1	576 colonies 0.25 mm. Clear hemolytic zones 0.75 mm.
	R. H., filtered	0.5	No growth
		0.25	30 colonies visible × 9. Non-hemolytic
		0.1	179 colonies 0.25 to 0.3 mm. Clear hemolytic zones 0.6 mm.
	Boiled 5 min.	0.5	428 colonies 0.8 to 1 mm. Clear hemolytic zones 3 mm.
	None		284 colonies 0.8 mm. Clear hemolytic zones 3 to 3.5 mm.

Experiment 2.—Culture F. C. from the throat of a milk handler and Scarlet Fever Streptococcus 55 were employed in this experiment. Milk in separate bottles was obtained directly from the right fore and right hind quarters of Cow 1462. It was chilled and freed of fat. One portion of each was filtered through Berkefeld candle V, another lot heated at 58°C. for 20 minutes, and the remainder boiled for 5 minutes. The various lots in amounts of 0.5, 0.25, and 0.1 cc. were incorporated in the plate cultures. The effect on the growth of both strains is recorded in Table II.

The results of Experiment 2 resemble those of Experiment 1. 0.5 cc. of milk, filtered or heated at 58°C. for 20 minutes to rid it of native bacteria, proved sufficient to inhibit or entirely suppress the growth of the scarlet fever streptococcus. In certain instances although colonies of the organism developed in the Petri dishes no hemolysis occurred; nevertheless when such colonies were subcultured and replated in the blood agar mixture characteristic hemolytic colonies developed. The control to which 0.5 cc. of boiled milk was added failed to show appreciable inhibition.

Two series of the plate cultures were photographed. In order to afford a proper comparison they were magnified about 5 times. Figs. 1 to 5 are the photographs of the plate cultures of Strain F. C. without milk (Fig. 1), with boiled milk (Fig. 2), and the series in which 0.5, 0.25, and 0.1 cc. of filtered milk was added (Figs. 3, 4, 5). Figs. 6 to 10 show the effect of mixing milk heated at 58°C. for 20 minutes with the cultures of Scarlet Fever 55. Photographs of the two controls, one without milk and the other with boiled milk, are included for comparison.

It might be argued that the milk from Cow 1462 contained some immune property acquired as the result of partial recovery from infection. To test this possibility Experiment 3 was devised.

Experiment 3.—Milk was obtained directly from the udder of five normal cows chosen at random from those of a large herd. It was mixed and when freed of fat a portion was filtered, another heated at 58°C. for 20 minutes, and a third lot boiled for 5 minutes. It was then added in the usual amounts to the Petri dishes and its effect on cultures of Strain F. C. and Scarlet Fever V noted.

It was found after 48 hours incubation that the growth of Culture F. C. in the plates containing 0.5 cc. of either filtered milk or milk heated at 58°C. was completely inhibited. In plates containing 0.25 cc. either nearly complete inhibition occurred, or the colonies were too small to be seen with the unaided eye. Even

as little as 0.1 cc. of milk greatly diminished the size of the colony and the zone of hemolysis. The same could be said of Scarlet Fever V. In both series the addition of 0.5 cc. of boiled milk failed to diminish the number or size of the colonies although the hemolytic zones were a little smaller than in the control cultures which were made without milk.

It appears certain then that there is a natural inhibitory substance in milk which passes through a Berkefeld filter V and is not greatly injured when milk is heated at 58°C. for 20 minutes but inhibits the growth of streptococci of the scarlet fever type in plate cultures. The inhibition depends on the concentration of the milk but even when this is diluted as much as 1:100, as in the series in which 0.1 cc. of milk was mixed with 10 or 12 cc. of agar and 0.5 cc. of blood, its effect is readily visible.

Since cow's milk contains a principle which is inhibitory even when mixed with the culture medium, it seemed probable that exposure of the streptococci to the direct action of undiluted milk might result in definite destruction. To test this point milk was inoculated with the streptococcus and plated, in the manner outlined by Jones and Little (1), in the hope that the rate of growth or destruction could be measured. As might be expected from the preceding experiments, milk even when inoculated with 3,000 or 4,000 streptococci per cc., failed to show growth when plated in amounts of 1 cc. It was necessary to change the methods considerably before decisive experiments were obtained.

Experiment 4.—Milk from five cows chosen at random was mixed and freed of fat by centrifugation. A portion was heated at 58°C. for 20 minutes and the remainder boiled for 5 minutes. Both lots were then distributed into sterile agglutination tubes in amounts of 1 cc. The tubes were then separated into two groups each containing an equal number of tubes of the pasteurized and the boiled milk. Each tube of one group was inoculated with 1 loop of Strain F. C. diluted 500 times in broth. Those of the other group received a similar inoculation with Scarlet Fever V. Plate cultures were made by adding 0.25 cc. of milk to uniform amounts of blood and agar. Initial plates were poured and others after various intervals of incubation. The results of this experiment are recorded in Table III.

While there is some irregularity in the results recorded in Table III, the influence of the milk is evident. It is clear that fresh milk heated to 58°C. for 20 minutes actually prevents multiplication of

scarlet fever streptococci during incubation periods ranging from 2 to 48 hours. From the protocol it appears that the milk probably destroyed both strains of streptococci after an interval of 4 or 6 hours. To determine whether this was actually the case required further experimentation. It seemed possible that the organism subjected to the unfavorable influence of the milk heated at 58°C. was incapable of growth when plated because of the fact that the culture medium

TABLE III.

The Effect of Undiluted Milk on the Scarlet Fever Streptococcus.

Culture	Milk	Colonies developing in plate cultures containing 0.25 cc. milk						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F C.	Heated 58°C.	No growth	243 Visible × 12	256 Visible × 12	No growth	No growth	No growth	No growth. Reaction pH 6.6
F. C.	Boiled	409	4,708 Whole plate hemolyzed	72,000	Innumerable	Innumerable	Innumerable	Coagulates on boiling. pH 5.2
Scarlet Fever 55	Heated 58°C.	384	154 Visible × 12, non-hemolytic	No growth	No growth	No growth	No growth	No growth. pH 6.6
Scarlet Fever 55	Boiled	512	7,488	86,400	Innumerable	Innumerable	Innumerable	Coagulates on boiling. pH 5.4

contained 0.25 cc. of the inhibiting milk. This factor, added to the effect of previous exposure, might still be insufficient to kill the organism although suppressing its multiplication. That the same milk when boiled for 5 minutes was well adapted as a culture medium is obvious in the protocol, since multiplication was noted throughout the series.

In the next experiment only a trace of milk was added to the plate cultures.

Experiment 5.—Milk was obtained from the same cows as in Experiment 4. It was handled in a similar manner. After distribution in agglutination tubes in amounts of 1 cc., each tube was inoculated with a loop of broth culture diluted 500 times. Each tube before plating was centrifuged at high speed for 15 min-

TABLE IV.

Streptococci Surviving after Incubation in Milk.

Culture	Milk	Colonies developing in plate cultures						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F. C.	Heated 58°C. 20 min.	205	5	11	11	Sterile	Sterile pH 6.6	Sterile pH 6.6
F. C.	Boiled 5 min.	218	8,960	86,400	Innumerable	Innumerable	Innumerable pH 6.0 Thickens on boiling	pH 5.4 Coagulates on boiling
Scarlet Fever V	Heated 58°C. 20 min.	217	205	192	205	77	11 pH 6.6	Sterile pH 6.6
Scarlet Fever V	Boiled 5 min.	218	4,992	46,080	Innumerable	Innumerable	Innumerable pH 6.0	pH 5.6 Coagulates on boiling

TABLE V.

The Effect of Milk on Scarlet Fever Streptococci at 3° and 4°C.

	Before refrigeration	After refrigeration	
		24 hrs.	48 hrs.
Culture F. C. in milk heated 58°C. 20 min.	205	24	12
" " " boiled milk	218	300	412
" Scarlet Fever V in milk heated 58°C. 20 min.	217	156	102
" " " " " boiled milk	218	130	166

utes. The bulk of the milk was drawn off and 1 cc. sterile salt solution added and thoroughly mixed. The mixture was then withdrawn and added to the Petri dishes. In this way it was hoped that so little milk would be added to the medium that its effect would be negligible. The majority of the tubes were incubated

and plates prepared at indicated intervals. Some of the tubes were refrigerated at 3–4°C. and their contents plated by the same method after 24 and 48 hour intervals. The colonies were counted after an incubation of 48 hours in the blood agar medium. The results are given in Tables IV and V.

It is evident that the scarlet fever streptococcus fails to multiply in mixed milk provided the milk has not been heated sufficiently to destroy the inhibitory substance. It is also true that the principle in milk actually destroys the organism. The lethal effect is most marked at 38°C. At this temperature Culture F. C. was killed after 8 hours incubation. Scarlet Fever Streptococcus V was more resistant to the action of milk, since there was a more gradual diminution in the number of organisms, but after 48 hours none survived.

When the same cultures were exposed to the action of milk in the refrigerator Strain F. C. again proved more susceptible since only about 10 per cent of the streptococci survived for 24 hours, whereas culture Scarlet Fever V was not appreciably affected by the milk during a refrigeration of 24 hours or even 48 hours.

When the experiment was repeated at the temperature of the room, the number of streptococci was definitely diminished although not so greatly as at incubator temperature. The surviving streptococci failed to approach in number those implanted in milk and refrigerated.

DISCUSSION.

It can be said that milk heated at 58°C. for 20 minutes or filtered through the coarsest Berkefeld filter possesses the property of inhibiting the growth of the scarlet fever streptococcus. It also is true that the principle is sufficiently active to destroy certain of the organisms. That this phenomenon cannot be attributed to the lack of adaptation of the organism for growth in the food mixture represented by milk is amply shown by the behavior when boiled milk is used. It must be recognized that this activity is not specific for the streptococcus since it has been shown by others that other types of organisms are inhibited. However, it appears to be particularly potent for streptococci especially those of the scarlet fever type. The inhibitory and lethal effect of milk on the streptococcus is most marked at temperatures approaching that of the body. When artificially infected milk is stored at 3° or 4°C. more of the streptococci survive.

Since the evidence points to the udder as the source of origin of the inhibitory principle it is not surprising that the action of the latter should be most effective at a temperature about that of the organ in which it originates.

Milk added to plate cultures entirely prevents the development of colonies or so changes the appearance of the colonies as to make them unrecognizable. In many instances signs of hemolysis are not to be seen. Even when as little as 0.1 cc. of milk is added to the medium the surrounding hemolytic zone is so small that the colonies can easily be mistaken for the bovine type. These facts must be borne in mind when mixed milk is examined for human hemolytic streptococci.

Since the experimental evidence indicates strongly that milk, provided it is not heated at too high a temperature, will inhibit the growth or kill the scarlet fever streptococcus, the opinion that severe outbreaks of scarlet fever result from human contamination of milk must be viewed with considerable doubt. It might be objected that the experiments were artificial. But they were optimum conditions for the growth of the streptococcus and other organisms which would rapidly sour the milk under natural conditions were excluded. The change from a broth medium to one containing milk would appear to be no greater than that from a human throat to raw milk. That a few individuals may contract the disease through direct human contamination of milk is possible, but the occurrence of epidemics would imply a heavy inoculation of the milk. Infection of the udder of a single cow with the scarlet fever streptococcus and the resultant shedding of large numbers of the organisms into the milk,—phenomena recorded in our foregoing papers,—afford a more reasonable explanation of milk-borne epidemics.

SUMMARY.

The experiments indicate that milk filtered through a Berkefeld candle V or heated at 58°C. for 20 minutes when added to blood agar plate cultures interferes with the development of colonies of the scarlet fever streptococcus. The observed inhibition is proportional to the amount of milk. When the approximate milk dilution in the Petri dish is 1:20 or 1:25 growth of the organisms is completely suppressed or only a small proportion of non-hemolytic colonies develop.

As the amount of milk is decreased the colonies become larger and their hemolytic zones more pronounced, although even when the final dilution of milk reaches 1:100 or 1:125 only colonies easily mistaken for the narrow zoned bovine streptococci appear. The effects upon the surviving organisms would appear to be transient since both the non-hemolytic colonies and those with small zones manifest the original hemolytic properties when transferred to other media. When scarlet fever streptococci are added in small quantities to milk heated at 58°C. for 20 minutes and incubated growth is inhibited. If the period is prolonged the streptococci are killed. On refrigeration of such mixtures some of the streptoccci are killed but others survive the test period.

BIBLIOGRAPHY.

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

EXPLANATION OF PLATE 38.

Magnification × about 5.

FIG. 1. Culture F. C. after 48 hours incubation in blood agar plate culture.

FIG. 2. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 3. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. filtered milk. There are no colonies of sufficient size to be detected at the magnification given.

FIG. 4. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.25 cc. filtered milk. Note the difference in size of colonies in this plate when compared with those in Figs. 1 and 2.

FIG. 5. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.1 cc. filtered milk. The colonies and hemolytic zones are larger than those in Fig. 4 but not as large as those in Figs. 1 and 2.

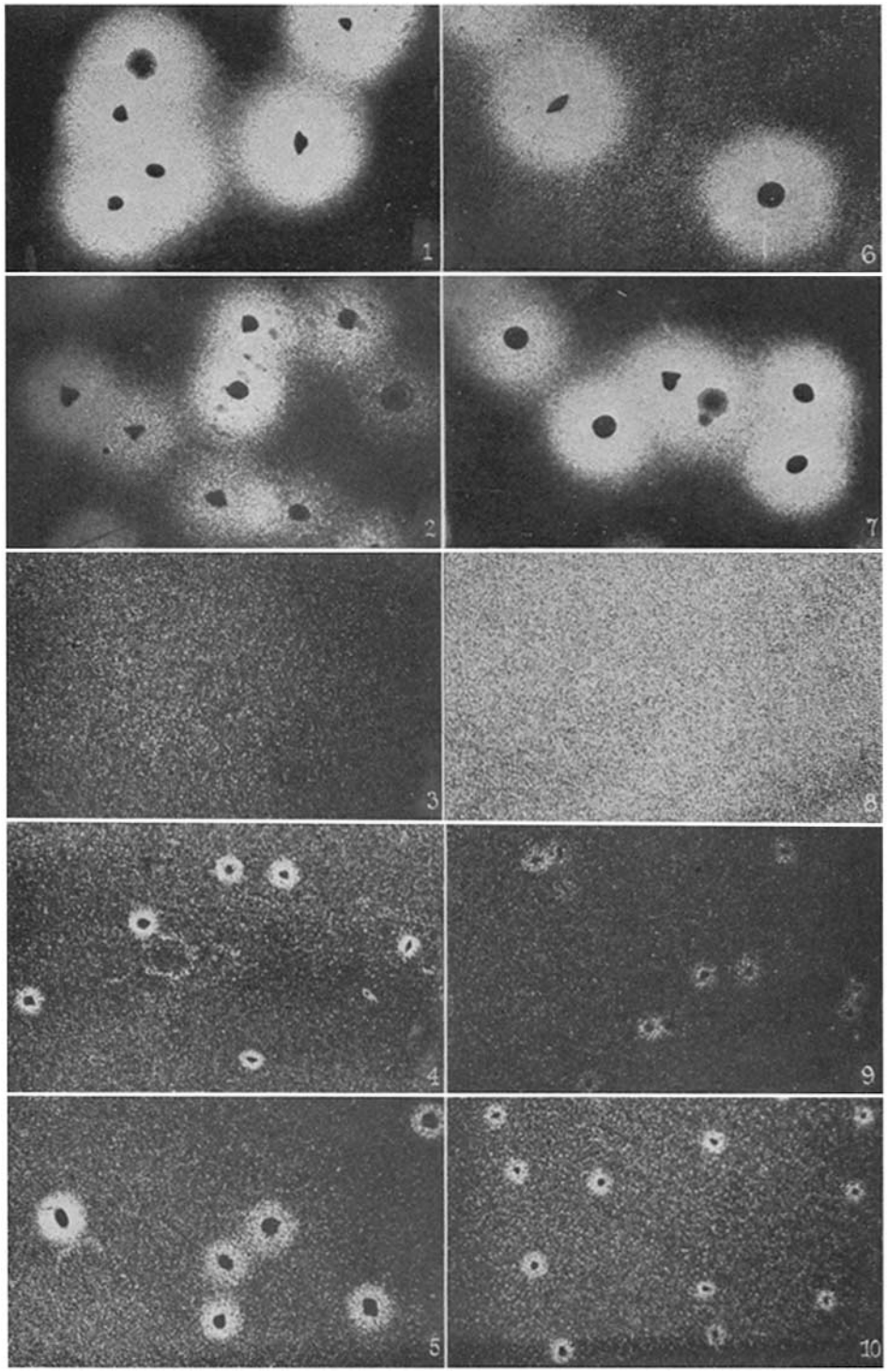
FIG. 6. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture.

FIG. 7. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 8. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. milk heated at 58°C. for 20 minutes. No growth visible at this magnification.

FIG. 9. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.25 cc. milk heated at 58°C. for 20 minutes. Note the small size of colonies and character of hemolytic zone as compared with Figs. 6 and 7.

FIG. 10. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.1 cc. milk heated at 58°C. for 20 minutes. The colonies and hemolytic zones are larger than those in Fig. 9 but not as large as those in Figs. 6 and 7.



Photographed by Louis Schmidt.

(Jones: Udder infection with streptococci. III.)