THE BACTERICIDAL PROPERTY OF COW'S MILK.

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Many have commented on the bactericidal properties of raw milk. It is well known that when certain bacteria are introduced into fresh raw milk their numbers decline for a time and subsequently multiply. Hesse[1] noted that *B. typhosus* and the cholera vibrio were prevented from multiplying in raw milk. Park[2] observed that the number of bacteria decreased in milk kept for 24 hours at 42°F., the same sample stored at 50°F. revealed a moderate increase in bacteria, and when the holding temperature reached that of the room or the incubator the bacterial increase was marked. Heinemann[3] found that milk contains for certain species of organisms a bactericidal substance, but the growth of other organisms is not inhibited. He further pointed out that the substance was destroyed at the boiling point or by heating to 60°C. for 30 minutes.

Rosenau[4] studied the phenomenon in considerable detail. He concluded that there is a diminution of the organisms in fresh raw milk and compared the action to that of a weak disinfectant and showed that the number of bacteria decreased markedly during the first 8 or 10 hours of incubation but rose rapidly thereafter. The action was prolonged but less rapid at 15°C. He attributes the apparent decrease largely to agglutination and calls attention to the possibility of phagocytosis by the leucocytes as an additional explanation for the decline.

More recently Chambers[5] showed that milk contains a definite bactericidal property which is destroyed by heating at 80° or 90°C. for 2 minutes. He found that the action was specific and depended on both the cow and the species of bacteria employed. He failed to find a relationship between agglutination and growth inhibition except that they were both destroyed by heat. It was pointed out that the lactic acid types of organisms were not inhibited in milk.

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[1] Hesse, W., *Z. Hyg. u. Infectionskrankh.*, 1894, xvii, 238.

[2] Park, W. H., *N. Y. Univ. Bull. Med. Sc.*, 1901, i, 71.

[3] Heinemann, P. G., The kinds of bacteria concerned in the souring of milk, Chicago, 1903.

[4] Rosenau, M. J., and McCoy, G. W., *U. S. Pub. Health and Marine Hosp. Service, Bull. 41*, 1908, 449.

[5] Chambers, W. H., *J. Bact.*, 1920, v, 527.

Sherman and Curran[6] inoculated fresh milk with young cultures of *Streptococcus lacticus*. There was lag in the fresh milk cultures for 30 minutes, but in the control tubes in which sterile milk was employed no lag occurred.

Hanssen[7] found that fresh raw milk would inhibit the growth of *B. typhosus* and *B. parathyphosus* B for 1 to 4 hours at 37°C. When the inoculated milk was kept at the temperature of the room the decrease was slower but more prolonged. He further showed that milk from single cows varied greatly in the inhibiting substance at different periods. This he attributes to variations in the ration, since he infers that the substance is due to the concentration of oxidizing enzymes in the milk. The enzymes supposedly originate in the food and their concentration in the milk is dependent on their concentration in the ration. He also showed that milk heated to 63°C. for 30 minutes and to 70°C. for 15 minutes still retained its inhibitory activity. 75°C. for 15 minutes inactivated the substance. This fact indicated to him that the substance in the milk was not derived from the alexin of the blood.

The phenomenon of bacterial decrease has been explained in several ways: lack of adaptation of the organisms employed (Stocking[8]), bacterial lag, agglutination-phagocytosis, and finally a definite property of fresh raw milk. A number of workers have failed to observe the phenomenon of growth inhibition in fresh raw milk.

If we should assume that milk possesses a definite bactericidal substance, perhaps its true purpose has been overlooked. Many have commented on its function, especially in regard to the keeping qualities of milk. That such is not its true function seems obvious. It is clear that the udder contains a relatively limited flora of organisms which have adapted themselves to their environment. Among the organisms usually encountered are streptococci and various types of micrococci; both are frequently associated with mastitis. It is also probable that many bacteria capable of multiplying in milk, such as the intestinal or vaginal flora, frequently come in contact with the ends of the teats; yet relatively rarely are organisms of this class encountered in the normal udder. When they occur they are usually associated with udder inflammation. Again, we are confronted with the problem of resistance to udder infection. Certain cows remain in the herd for several years without developing udder disease while

[6] Sherman, J. M., and Curran, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 15.

[7] Hanssen, F. S., *Brit. J. Exp. Path.*, 1924, v, 271.

[8] Stocking, W. A., *Rep. Conn. Agric. Exp. Station*, Storrs, 1904, 89.

others suffer from repeated attacks. As far as known, the blood and milk of the resistant group contain no more agglutinins than the same fluids of the susceptible animals. That the bactericidal substance in milk might be of considerable assistance in preventing the growth of organisms within the udder seemed worthy of investigation, particularly if it would throw light on the problem of resistance to mastitis. With these points in view a number of observations were made.

EXPERIMENTAL.

Methods.—Since our problem concerned itself with the possible influence of the inhibitory substance within the udder, we decided to test the milk against the non-hemolytic mastitis streptococcus, the organism we have found most frequently in udder infection. Our Culture 55 was isolated in 1917 and since that time has been carried on horse blood agar or plain agar slants. It is usually transferred four or five times a year. Presumably it has adapted itself to growth on artificial media but readily multiplies in milk. It has the further advantage of agglutinating slowly even with high titered agglutinin.

Milk was always drawn directly into sterile bottles and all precautions were taken to avoid contamination. Generally the samples were taken from the middle portions of the milking. The milk after chilling was rapidly centrifuged and a portion of the liquid between the cream line and sediment withdrawn. In this way a relatively fat-free milk containing very few cells and a limited number of organisms was obtained. The milk was then distributed in amounts of 1 cc. in sterile agglutination tubes containing a glass bead. A sample of boiled or autoclaved milk was also distributed in the same way for control purposes.

All tubes were then inoculated with one loop of an 18 hour broth culture of the mastitis streptococcus, which was diluted 100 times with broth. Each tube was shaken before incubation and at half-hourly intervals throughout the experiment. The contents of the tubes were withdrawn at various times and plated with 12 cc. of 2 per cent agar prepared from veal infusion. The number of colonies was counted after 24 hours incubation at 38°C.

Our methods largely eliminated the cells present in the milk as well as decreased the number of udder organisms. The repeated shaking of the tubes containing the glass beads prevented agglutination, since clumps could not be detected on microscopic examination.

When blood serum was used it was handled in a similar manner. It was always freshly obtained and relatively free from cells.

Experiment 1.—In the herd to which we have had access, we had the opportunity of testing the bactericidal action of the milk from four groups of cows. The first group consisted of native cows which had been in the herd for several years and had no history of mastitis. The second group had been injected on four occasions with killed cultures of the mastitis streptococcus. They had been recently im-

TABLE I.

The Bactericidal Activity of the Milk and Serum of 8 Cows for the Non-Hemolytic Mastitis Streptococcus.

Cow No.	Group	Milk + culture of mastitis streptococcus				Blood serum + culture of mastitis streptococcus				
		Number of colonies				Number of colonies				
		At once	After 2 hrs.	After 4 hrs.	After 8 hrs.	At once	After 2 hrs.	After 4 hrs.	After 8 hrs.	After 20 hrs.
1	Resistant	8,448	6,464	6,784	6,400	8,704	7,040	6,336	4,864	2,688
2	"	8,960	8,320	6,528	6,218	8,448	8,768	6,786	2,592	42
Sterilized milk	Control	9,344	24,768	Innumerable	Innumerable					
3	Vaccinated	4,928	4,540	3,520	20,100	5,120	5,824	4,480	4,350	Innumerable
4	"	4,740	3,710	3,392	22,000	4,992	5,760	5,120	2,560	41,600
Sterilized milk	Control	5,370	9,792	Innumerable	Innumerable					
5	Young cows in first	4,480	3,520	3,584	28,800	4,480	3,456	4,160	3,648	Innumerable
6	lactation period	4,480	3,390	4,800	Innumerable	4,160	3,748	3,712	4,544	"
Sterilized milk	Control	4,860	9,090	Innumerable	"					
7	Cows susceptible	4,416	3,588	3,584	"	4,416	4,864	4,544	5,376	"
8	to mastitis	4,544	3,904	3,392	"	4,288	4,991	3,776	2,240	"
Sterilized milk	Control	4,352	10,760	Innumerable	"					

ported from Tennessee and their udders were free from non-hemolytic streptococci when introduced into the herd. The third group consisted of young native animals in their first lactation period. The fourth series were animals which had had several attacks of mastitis. The findings were about the same for all the animals in each group so that only the details of two tests are given. The details of the results of the bactericidal activity of both the milk and blood serum are given in Table I. In these observations sterilized milk from the laboratory supply was used for control purposes.

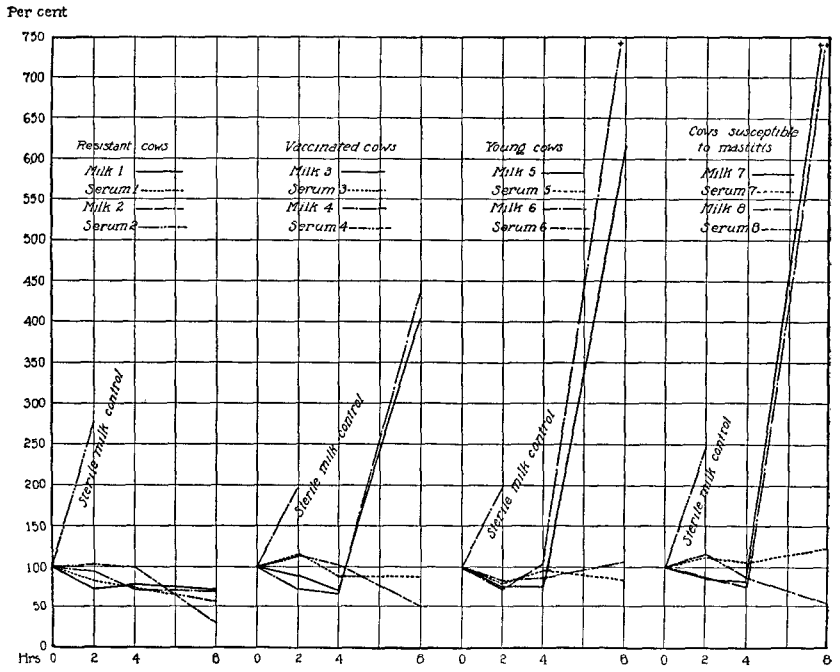

CHART 1. The inhibition of the mastitis streptococcus in milk and blood serum of resistant, vaccinated, and young cows and of cows susceptible to mastitis.

The data given in Table I have been plotted for the purpose of comparison and are shown in Chart 1. In the charts the number of colonies counted in the samples of both the raw and the boiled milk in each series have been added and an average struck; this represents 100 per cent, and departures from this line are recorded in terms of per cent. The results with the serum are given in the same way.

Both the table and the chart illustrate certain features. The milk

from all groups reacts in a similar manner. There is a sharp decline in the number of organisms during the first 4 hours of incubation, which is common to all groups. After this time there is a rapid multiplication particularly in the instance of the young cows and those susceptible to mastitis. The vaccinated group are about midway between the most resistant animals and the young and susceptible cows. The inhibition of the milk of the resistant cows is marked, since there is no multiplication during the first 8 hours. In sharp contrast is the rapid increase in the number of streptococci in the sterilized milk control. Here the number of organisms nearly doubled during the first 2 hours.

TABLE II.

The Bactericidal Activity of the Milk of a Resistant Cow and of a Young Cow Early in Its First Lactation Period.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk of resistant Cow 1	5,200	4,392	4,928	4,608	12,608
Boiled milk of Cow 1	5,000	48,900	Innumerable	Innumerable	Innumerable
Milk of young Cow 9, early in first lactation period	5,356	4,492	4,262	6,220	22,464
Boiled milk of Cow 9	5,068	36,990	Innumerable	Innumerable	Innumerable

It will be noted that the serum was in all cases inhibitory during 8 hours. This in itself is of little significance since the organism has not been trained to grow in serum and hence the lag period is relatively long. However, when the incubation period was increased to 20 hours, it was found that the serum of resistant Cows 1 and 2 was distinctly bactericidal and that of Cow 4 had definite inhibitory properties.

Inasmuch as in these observations both the milk and the blood were obtained in the morning and tested during the day, no opportunity was afforded for observations in the 6th hour, perhaps a critical point. This point has been covered in Experiment 2.

Experiment 2.—The milk of resistant Cow 1 and a young cow early in the first lactation period was obtained and employed in the manner outlined. In this and

ensuing observations the control consisted of a portion of the milk boiled for 5 minutes. The data have been tabulated in Table II.

From Table II it is clear that the inhibitory substance may be about as active in the milk of a young cow early in its first lactation period as that manifested in the secretion of an old cow which has passed through several lactation periods.

The behavior of the streptococcus in boiled milk affords a sharper contrast than the use of the stock milk medium which has been autoclaved. The former apparently represents an ideal medium, since it is the fresh product boiled for 5 minutes. The stock medium is prepared from commercial skim milk from the same dairy; it is older when separated and requires titration. It is of course heated to a higher temperature for a longer period.

Thus far it has been shown that the bactericidal substance is present to a varying degree in the milk of all cows examined. In addition to these reported, the milk from ten others has been examined with practically identical results. There is always a period from 4 to 8 hours in which the streptococcus fails to multiply. We are inclined to the opinion that during this period there is actual diminution succeeded by a period of multiplication. The streptococcus has thus far failed to coagulate the raw milk before the 8th hour, and in samples possessing strong inhibitory activity there is no coagulation at the end of 24 hours. From a number of observations we are convinced that most of the inhibitory substance is utilized during the first 8 hours, since as soon as the streptococcus begins to increase it continues to do so at a rapid rate. This is well borne out in Table I. Both the boiled milk and the sterilized milk controls are always definitely thickened at 8 hours and often firmly coagulated.

From Table I it appears as if there was a parallel between the activity of the blood serum and that of the milk; it seemed likely that the substance originated in the blood and appeared in a diluted form in the udder. One or two points, however, indicate that this explanation is not entirely true. If the substance originated in the blood, it might be possible to increase its concentration in the milk by immunization. The injection of dead cultures of streptococci into the cows of Group 2 (Table I) did not increase the concentration in the udder

above the usual level. The milk of young cows early in their first period of lactation compared in its inhibitory effect with that from the

TABLE III.

The Inhibitory Effect of Milk on Culture 55 and on a Freshly Isolated Strain of Mastitis Streptococci.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk of resistant Cow 1 +Culture 55	6,336	4,800	5,120	6,720	9,185
Boiled milk of Cow 1 + Culture 55	7,260	20,160	Innumerable	Innumerable	Innumerable
Milk of Cow 1 + freshly isolated streptococci	7,168	5,788	6,912	5,760	41,520
Boiled milk of Cow 1 + freshly isolated streptococci	7,296	16,588	Innumerable	Innumerable	Innumerable
Milk of young Cow 10, early in first lactation period + Culture 55	5,356	4,492	4,262	6,220	22,464
Boiled milk of Cow 10 +Culture 55	5,068	36,992	Innumerable	Innumerable	Innumerable
Raw milk of Cow 10 + freshly isolated streptococci	1,344	1,241	1,088	11,980	19,120
Boiled milk of Cow 10 + freshly isolated streptococci	1,197	30,538	Innumerable	Innumerable	Innumerable

others. It is to be presumed that these cows had been exposed to infection for too short a period to acquire resistance to the mastitis streptococcus.

Since we were convinced that the raw milk of all the cows contained a definite substance which inhibited the growth of the streptococcus, we decided to continue the study with the hope of throwing some light on its properties and its probable source. In Experiment 3 we attempted to show the influence of the culture on the inhibitory substance. The inhibition against Culture 55, previously referred to, and a freshly isolated strain of the mastitis streptococcus in the second culture generation were compared. The results are recorded in Table III.

It is apparent that the inhibitory substance in the milk of Cow 1 is a little more effective against the culture which has been carried on artificial media for a longer period than against the freshly isolated strain, although in the latter instance there is well marked inhibition. In the case of Cow 10, a young animal early in the first lactation period, the effect of the addition of the freshly isolated streptococcus is more pronounced, for inhibition occurred only within the first 4 hours. The experiment also indicates that the freshly isolated strains are better adapted for growth in the raw milk than the older strain isolated several years ago and since cultivated on artificial media.

Although the growth-inhibiting effect of whole or skim milk has been commented on by many, we have failed to find any reference whether the agent is contained in the whey. To test the effect of whey a number of experiments were made. Experiment 4 affords an example.

Experiment 4.—Commercial rennet tablets were ground and dissolved in sterile salt solution in the proportion of 2 tablets to 20 cc. of salt solution. The solution was sterilized by filtration through a Berkefeld candle N. 2.5 cc. of the filtrate was added to 50 cc. of fresh milk largely freed from fat, and after mixing was placed in a water bath at 38°C. Within 10 or 15 minutes sufficient whey was obtained for the tests. As a control procedure 2.5 cc. of salt solution was added to 50 cc. of the milk and the mixture heated in the water bath at 38°C. A third portion of the milk was boiled for 5 minutes. The whey, raw milk, and boiled milk were distributed in the usual manner and inoculated with the culture. The inhibitory effect of the whey and of the milk is given in Table IV.

It is apparent that whey contains the inhibitory substance to about the same extent as the milk. It cannot be said that the substance is greatly concentrated by the removal of the casein.

It has been shown by one of us[9] that milk will react to a relatively uniform level with a specific cow serum precipitin. The milk of Cow 1 used in this experiment reacted with cow serum precipitin at a maximum dilution of 1:640. The whey reacted at a dilution of 1:2,560. Therefore, in the process of rennet coagulation there is a considerable concentration of blood protein in whey. If the inhibitory agent comes from the blood then it is to be expected that it would be considerably concentrated within the whey. The protocols submitted in Table IV show that this is not the case.

Chambers demonstrated that 80° or 90°C. for 2 minutes would destroy the bactericidal effect of milk. Heinemann stated that

TABLE IV.

A Comparison of the Inhibitory Effect of Raw Milk and of Whey on the Mastitis Streptococcus.

	Streptococci present					
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 20 hrs.
Milk of Cow 1	5,200	4,392	4,928	4,608	12,608	Innumerable
Whey of Cow 1	5,440	4,224	4,608	4,032	8,896	"
Milk of Cow 11	5,440	4,160	5,632	5,760	28,800	"
Whey of Cow 11	5,696	5,440	4,352	8,064	28,224	"
Combined milk of both cows, boiled for 5 min.	5,000	48,960	Innumerable	Innumerable	Innumerable	"

60°C. for 30 minutes would destroy the substance. Rosenau claimed that the inhibitory effect for *B. lactis aerogenes* was weakened at 55°C. and almost destroyed at 60°C. On the other hand, 60°C. did not affect the inhibition against typhoid. Hanssen states that the inhibitory substance for *B. typhosus* resists a temperature of 63°C. for 30 minutes and 70°C. for 15 minutes, although it is inactivated at 75°C. for 15 minutes. Since there is some difference of opinion in regard to the temperature required to inactivate or destroy the substance in milk, it was desirable to try the effect of various temperatures. For purposes of comparison we also observed the effect of

[9] Jones, F. S., *J. Exp. Med.*, 1926, xliii, 451.

the same temperatures on a cow blood serum of considerable bactericidal activity. It also seemed to us that if it was possible to show that the substance in milk was more or less resistant to heat than that of the blood, it would be regarded as additional evidence that they were different substances. That this really proved to be the case is brought out in Experiment 5.

Experiment 5.—Milk from Cow 13 which would inhibit growth of the mastitis streptococcus during the first 8 hours of incubation was obtained as usual. It was distributed in tubes in amounts of 10 cc. The contents of one tube was used in its raw state, another tube was boiled for 5 minutes, and the others were heated for 20 minutes at temperatures of 56°, 58°, 60°, 62°, 65°, 70°, and 80°C. The milk was then cooled and distributed in amounts of 1 cc. in the small tubes containing glass beads and inoculated with the culture. Cow 1 was bled in the afternoon and the blood stored in the refrigerator overnight. The serum was clear and free from cells. It was distributed in amounts of 7 cc. One tube was not heated. The others were heated for 20 minutes in the water bath at temperatures of 56°, 58°, 60°, 62°, and 65°C., and then all were inoculated with the culture. In this experiment we used a strain of the streptococcus which had been rapidly passed through broth which contained 50 per cent cow serum heated at 60°C. for 30 minutes. The culture was of the same age as the one used to inoculate the milk. The data are given in Table V.

It is apparent that the bactericidal factor for the mastitis streptococcus in the blood serum is inactivated at 56°C. With the increase in temperature inhibition of the substance is more marked. The serum heated at temperatures from 56° to 62°C. reveals no gross changes, but at 65°C. it becomes slightly opalescent; above 65°C. it coagulates. In contrast is the higher temperature required to inactivate the inhibitory agent in milk; temperatures ranging from 56° to 62°C. fail to affect it. When the temperature was increased to 65° or 70°C. for 20 minutes there was some impairment, although milk so treated proved inhibitory during the first 6 hours. 80°C. for 20 minutes is as efficient in inactivating the substance as boiling for 5 minutes.

The difference in the temperature required to inactivate the bactericidal substance in blood serum and in milk strongly suggests that the substances are different.

It is well known that antibody and blood proteins pass from the

TABLE V.

The Effect of Various Temperatures on the Bactericidal Activity of Milk and Cow Serum.

	Milk					Blood serum					
	Number of colonies					Number of colonies					
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 20 hrs.
Unheated	4,352	5,584	3,008	2,304	3,456	8,000	6,592	7,168	5,248	5,056	6,720
Heated 20 min. at 56°C.	4,736	3,328	3,136	2,560	3,136	7,424	8,064	31,100	52,000	Innumerable	Innumerable
58°C.	4,480	3,200	2,560	3,136	3,392	8,064	9,600	48,900	Innumerable	"	"
60°C.	4,160	3,776	2,624	3,072	3,264	7,616	13,760	86,000	"	"	"
62°C.	4,480	3,072	2,626	2,944	3,392	8,000	17,920	Innumerable	"	"	"
65°C.	4,608	3,072	2,368	2,880	115,200	7,268	40,300	"	"	"	"
70°C.	4,608	3,072	3,328	3,072	115,200						
80°C.	3,264	46,000	Innumerable	Innumerable							
Boiled 5 min.	4,352	57,600	"	"							

milk into the blood. Ehrlich and Wassermann[10] noted that the milk contained from 1/15 to 1/30 as much antitoxin as the blood serum. Osborne and Wakeman[11] by analysis obtained 0.2 gm. of lactoglobulin per liter of milk. Crowther and Raistrick[12] and Wells and Osborne[13] were unable to distinguish lactoglobulin from serum globulin by chemical or anaphylactic methods. Howe[14] has shown that the blood of 30 months old cows contains on an average 5.06 gm. of globulin per liter. If, then, the dilute products of blood serum are responsible for inhibition, a medium containing about 1/25 part of fresh blood serum should prove inhibitory. That such is not the case is brought out in the following experiment.

Experiment 6.—The following medium was devised as a substitute for milk. Casein, 2.5 gm.; N/20 NaOH, 40 cc.; 0.9 per cent NaCl solution, 60 cc.; lactose, 5 gm. Autoclaved for 15 minutes at 15 pounds pressure. The reaction was pH 7.4. To 25 cc. of the above 1 cc. of sterile fresh serum from Cow 1 was added. This was distributed into small tubes and inoculated with the streptococcus. As a control procedure the medium which contained no serum was also inoculated with the culture. Plates were prepared from both series at once and after 2, 4, 6, and 8 hours incubation. As far as we could determine, growth took place to about the same degree in the medium containing serum as in the one which did not. There was an increase during the first 2 hours and a steady, though not rapid, increase thereafter.

It is true that an amount of serum proportionate to the globulin content of normal milk is insufficient to inhibit the growth of the non-hemolytic streptococcus in a relatively poor synthetic medium.

Rosenau has commented on the relative specificity of the reaction in milk since one of his samples restrained the typhoid bacillus and staphylococcus but not Parathyphoid A and B. In the sense that Rosenau used the term "specific" it is probably true, since in our hands milk which would restrain the streptococcus had little effect on one strain of *B. coli* and only a moderate restraining influence on *B. bovisepticus.*

[10] Ehrlich, P., and Wassermann, A., *Z. Hyg. u. Infectionskrankh.*, 1894, xviii, 239.

[11] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xxxiii, 7.

[12] Crowther, C., and Raistrick, H., *Biochem. J.*, 1916, x, 434.

[13] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1921, xxix, 200.

[14] Howe, P. E., *J. Biol. Chem.*, 1922, liii, 479.

Thus far there has been no evidence submitted to show whether the multiplication of one species of organism will absorb the inhibitory substance for another. With this in view the following experiment was devised.

Experiment 7.—Milk from Cow 13 was collected as usual and after centrifuging was divided into lots of 10 cc. Lot 1 was refrigerated at once; Lot 2 was heated to 62°C.; to each 10 cc. of Lot 3 the growth from 5 cc. of an 18 hour broth culture of the streptococcus was added; and Lot 4 was treated in the same way except that each 10 cc. received the growth from 7.5 cc. of broth culture of *B. bovisepticus*. Another portion of the milk was boiled for 5 minutes. The tube heated at 62°C. for 20 minutes and the tubes to which the cultures were added were incubated at 38°C. for 6 hours. The tubes inoculated with the cultures were then heated at

TABLE VI.

The Effect of Absorption with Streptococci and B. bovisepticus on the Inhibitory Substance in Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Raw milk, control	2,752	2,688	2,432	2,304	3,264
Milk heated at 62°C.	2,752	3,008	2,688	2,120	2,814
Absorbed with Streptococcus 55	3,200	4,736	Innumerable	Innumerable	Innumerable
Absorbed with *B. bovisepticus*	2,816	3,264	14,848	"	"
Boiled milk control	2,752	29,952	Innumerable	"	"

62°C. for 20 minutes. Exposure at this temperature was sufficient to kill the organisms but not inactivate the inhibitory property. It was necessary to adjust the reaction to the original pH of the milk of Lots 3 and 4 with N/Na_2CO_3. The effect of the absorption and heat was then tested in the usual manner after the addition of the mastitis streptococcus. The results are seen in Table VI.

Under the conditions it is apparent that the growth of one species of organism will absorb the inhibitory principle for another. It was not possible to completely absorb the substance, it is true, but the fact that for this purpose the strain of *B. bovisepticus* was about as efficient as the streptococcus argues that the substance is not specific for only the streptococcus.

We supposed from the frequent reference by other workers to fresh milk that this bactericidal substance deteriorated rapidly, even when kept at favorable temperatures. On several occasions we tested milk that had been stored at 6°C. for 2 or 3 days and found the efficiency of the inhibitory element to be unimpaired. After 10 days we failed to find any difference. After refrigeration for 27 days the milk was still capable of inhibiting the growth of the streptococcus for 4 hours with slow growth from this time onward. Originally it would inhibit 8 hours. The sample held for a period of 2 months contained a large number of putrefactive organisms and could not be tested.

DISCUSSION.

It is apparent that there is present in the milk of all the cows examined a substance which will inhibit the growth of mastitis streptococci for a definite period. The concentration of the inhibitory substance varies in the secretion of different animals. It may be as concentrated in a young cow early in its first lactation period as in an older, more resistant animal which has been repeatedly exposed to infection with streptococci. It is equally true that artificial immunization with killed cultures did not lead to an appreciable increase of the inhibiting agent in the milk. The inference is that the substance is a natural one since it is not increased by exposure to streptococcic infection or artificial immunization and because it is not specific for a single organism. The fact that the milk of young cows early in the first lactation period contains the substance in about the same amount as the older animals argues that it is inherent.

Whether or not such a substance under the present conditions of dairying may be regarded as of much value in protecting the udder against the rapid multiplication of bacteria which have gained entrance is open to question. However, under the most favorable conditions it is possible that a few organisms which have recently gained access to the udder might be prevented from multiplying until they are flushed out at the next milking. It may be that the substance is more potent than we suspect, since many opportunities are available for the entrance of bacteria into the teat canal although in the main the flora of the udder is limited to relatively few species and these

species have become well adapted to the environment and the inhibitory influence of this substance.

Under more natural conditions the substance might have considerable influence in protecting the udder. From the experiments the inhibition of growth of the mastitis streptococcus during the first 4, 6, or 8 hours was strong. Under natural conditions the calf would empty the udder at intervals which correspond to these periods. The inhibiting agent would act to prevent bacterial multiplication and thus insure milk of low bacterial content for the calf. The lengthening of the period between milking to 12 hours under the usual conditions of dairying has more or less rendered the substance inoperative.

Although we have no data directly bearing on the origin of the substance, by inference it seems possible to localize its source. Rosenau's contention that the diminution is due to agglutination is probably true for the organisms he tested. Our technique seems to rule out agglutinin, especially in view of the microorganism employed. The blood contains only weak agglutinin for the streptococcus, which is not easily agglutinated, and clumps could not be detected on microscopic examination of the milk.

If the substance is of blood origin the inhibitory substance should be greatly increased in the whey since with rennet coagulation there is a considerable concentration of the blood proteins, as shown by serum precipitin tests of the whey.

The view that the substance is "alexin" from the blood is not supported by our observations. Thus, in the normal udder there is only a slight interchange of blood proteins from the circulation to the milk and the concentration is too low to be a serious factor. This fact is borne out by the experiment in which fresh blood serum was added in the proportion of 1:25 to a special medium containing casein and lactose without inhibiting the growth of the streptococcus. The alexin from the blood of cows, like that of most mammals, is inactivated at a temperature of 56°C. for 20 minutes, while milk containing the inhibitory substance may be heated for the same period at 62°C. without impairing its inhibitory properties. It is not completely inactivated at 65° and 70°C. In this respect, then, there is a distinct difference between the substance in the milk and that of the blood. We infer from these facts that the substance originates in the udder.

It however resembles alexin in that it deteriorates slowly even at a temperature of 6°C.

Hanssen's contention that the substance is an oxidizing enzyme must be given consideration. He noted that the inhibition was more marked in the cow on pasture than in the milk of the same cow during the winter. From this he inferred that the substances originated in the food. The cows in our observations were fed about the same ration throughout the year.

Our experiments further indicate that the action of the substance is not specific, since by incubating raw milk with one organism we were able to absorb the inhibiting agent for another.

SUMMARY.

The bactericidal activity of fresh raw milk from a number of cows has been tested with the non-hemolytic mastitis streptococcus. By using this organism and other means we were able to rule out the action of agglutinin. The milk of all cows examined inhibited the growth of the streptococcus for definite periods. The length of the inhibition period varied; the milk from some cows prevented growth for 8 hours, that of others for only 4 or 6 hours. The inhibitory action may be as strong in the milk of a young cow in its first lactation period as in that of an old cow known to be resistant to udder infection. It is possible to absorb the streptococcus inhibitory substance by first inoculating the milk with *B. bovisepticus*. We were unable to show that the substance was increased by artificial immunization of cows with the streptococcus.

Whey obtained by the action of sterile rennet solution inhibited the growth of the streptococcus to about the same extent as the milk from which it was obtained.

We infer that the substance originates in the udder since it differs from blood alexin in its resistance to heat, it is not increased in the whey although the blood proteins are more concentrated, and it is not increased in the milk when the cows are artificially immunized or repeatedly exposed to natural infection.