THE BACTERICIDAL ACTIVITY OF BOVINE BLOOD FOR BRUCELLA

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE

Evelyn Marie Wood

1944

This is to certify that the

thesis entitled

Bactericidal Action of Bovine Blood toward Brucella.

presented by

Evelyn Wood.

has been accepted towards fulfilment
of the requirements for

M.S degree in Bacteriology

J. Howard Brueckner
Major professor

Date 2/16/44

THE BACTERICIDAL ACTIVITY OF BOVINE BLOOD FOR BRUCELLA

by

Evelyn Marie Wood

A THESIS

Submitted to the Graduate School of Michigan State College of Agriculture and Applied Science in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology

February, 1944

ACKNOWLEDGMENT

I wish to thank Dr. I. F. Huddleson for suggestions in this project, and Dr. G. C. Richardson for veterinary assistance.

The Bactericidal Activity of Bovine Blood for Brucella

THE BACTERICIDAL ACTIVITY OF BOVINE BLOOD FOR BRUCELLA.

The bactericidal property of blood has been under extensive study since the early work of Nuttal (11) in 1888. It is a more complex subject than generally supposed and its significance not easily interpreted. At present it is impossible to bring the results of different workers, who have investigated bactericidins, into harmony and draw general conclusions because of the differences in the methods of study. However, certain variables in the bactericidial capacity of normal serum have been established. Mackie and Finkelstein (6) have made a comprehensive study of the occurrence of bactericidins in the blood serum of several species of animals, and the susceptibility of different bacteria to their action. They found that the bactericidal power of blood for a given microörganism varied in different species of animals and in individual animals of the same species. Furthermore, different bacteria react differently in their susceptibility to the bactericidins.

*This study represents a cooperative project of the Bacteriological Section of the Michigan State Agricultural Experimental Station and the Bureau of Animal Industry of the United States Department of Agriculture. The work was carried out in the Brucellosis Laboratory of the Department of Bacteriology.

The workers just mentioned included the three species of Brucella in their studies of serums from sheep, ox, human, rat, pig, horse, rabbit, guinea pig and pigeon. They found that serums from all the species possessed bactericidal action towards Brucella. However, the comparative bactericidal effect of the various serums and the difference in susceptibility of the three species of Brucella to their action, were not accurately measured. The method used by Mackie and Finkelstein was not one of quantitative measurement. The bactericidal effect was determined by loop transfers (before and after incubation) from each mixture to a plate of culture medium. The bactericidal effect was estimated by comparison of the end-points of growth in the test and control series. No bactericidal action was noted until after 20 to 24 hours of incubation.

Mackie and Finkelstein (6) attribute the bactericidal property of normal serum towards Gram-negative bacteria to a "thermolabile" bactericidin consisting of complement and a sensitizing antibody. While the existence of certain natural bactericidal antibodies in normal serum has been proved by Muir and Browning (8), there has been no clear evidence to show that the bactericidal property of normal serum depends entirely on bactericidal antibody.

Irwin and associates (5) were the first to test the susceptibility of the genus Brucella to the bactericidal action of bovine blood. These workers found that bovine whole blood, serum, and plasma exhibited slight bactericidal action for Brucella. Br. abortus was found more susceptible to the action of bactericidins than Br. suis.

Striedter and Avrukina (14) observed that Br. melitensis was relatively more resistant to the bactericidins in serum of guinea pigs and sheep than was Br. abortus.

The immediate objectives in this study, were (1) the development of a controllable bactericidal system which would detect the occurrence of bactericidins in bovine blood serum or plasma, (2) the employment of the system in following the changes, if any, that occurred in the blood of calves from birth to maturity, in immunized animals and in animals infected with Brucella.

While the development of a satisfactory bactericidal system was in progress, it was noted that many factors influenced the end results. A few of these were, (1) period of incubation, (2) number of organisms used, (4) dissociation of the organism, (5) interval between collecting blood and setting up the test, (6) the use of serum, plasma or whole blood, (7) filtration of serum or plasma and the type of filter used, (8) heating of serum or plasma.

A considerable portion of the Thesis is devoted to a study of the factors which influence the results of a bactericidal system.

EXPERIMENTAL

Materials and Methods:

Preparation of culture: A single, smooth culture of Br. abortus was used unless otherwise stated. The microorganism was grown on beef liver agar slants for 48 hours at 37°C. The growth was removed by means of a sterile wire-loop and suspended in sterile diluting fluid which consisted of 0.05 per cent Tryptose peptone and 0.5 per cent NaCl in distilled water. The bacterial suspension was thoroughly mixed and then diluted to a scale reading of 23 on the Libby Photronreflectometer. The standard suspension contained from 800,000,000 to 1,300,000,000 live Brucella organisms per ml. Serial dilutions ranging from 1:100 through 1:1,000,000 were made from this suspension. The number of bacteria present was determined by floating 1 ml. of the 1:1,000,000 dilution over the surface of a Tryptose agar plate. The Tryptose agar contained crystal violet in a 1:1,000,000 dilution. The plates were incubated at 37°C for four days. The number of colonies counted was recorded as representing the initial number of bacteria added to the blood plasma or serum from the different dilutions.

Preparation of blood: Blood was drawn asceptically from the jugular vein of the animal and collected in sterile bottles. For the test requiring whole blood or plasma a sufficient amount of sterile saturated sodium citrate was placed in each bottle to prevent the blood from clotting. Whole blood, plasma, or serum samples that were not used within a few hours after collection

were refrigerated at 4°C. When plasma or serum was used, the blood was centrifuged for thirty minutes and the supernatent drawn off aseptically. The serum or plasma samples were collected from normal animals as regards to Brucella unless otherwise stated.

Experiments and Results:

I. Determination of incubation period: In establishing a standardized procedure, the incubation period necessary to attain the maximum bactericidal effect was sought. Five duplicate series of plasma-bacterial suspensions and controls were made as follows: Into each of a series of sterile, cotton plugged, 10 ml shell vials was pipetted 1 ml of sterile diluting fluid. To the first tube, 1 ml of plasma was added, and mixed with a 1 ml syringe. Then, 1 ml of the mixture was transfered to the next tube, and thus continued until the desired number of plasma dilutions were obtained. To each vial, 1 ml of a 1:1,000,000 dilution of the standard suspension of organisms was added. The plasma-bacterial suspension was then thoroughly mixed, and placed at 37°C. Each duplicate series was removed from the incubator at intervals of 2, 4, 8, 10, and 24 hours, and plated on Tryptose agar. The results obtained are recorded in Table I. The results show that the bactericidal action of bovine plasma is not one of abrupt killing, but a gradual process, dependent upon the time of incubation and the ratio of plasma

Table I

The bactericidal activity of plasma after variable periods of incubation.

Plasma dilutions	Periods of incubation at 37°C				
	2 hours	4 hours	8 hours	10 hours	24 hours
	Colony counts				
1:2	1164	850	280	108	42
1:4	1180	978	230	160	42
1:8	1230	1198	512	256	208
1:16	1254	1260	972	426	164
1:32	1257	1262	1076	820	744
1:64	1400	1270	1254	1082	1180
1:128	1874	1400	1400	1200	6874
Control*	1500	1700	2000	2400	12000

*The number of bacteria added = 1.2×10^{5}.
*The number of bacteria after incubation.

to the number of organisms. In the two highest dilutions of plasma, the organisms multiply during the first 2 hours, and then show a slight decrease in number during the next 10 hours. After the 24th hour an increase in numbers takes place. Evidently the bactericidins are too weak in concentration to prevent growth. The absorption of a minute amount of bactericidins by the bacteria apparently has a bacteriostatic action and only a slight bactericidal action.

Later, it was observed that when a large number of bacteria were added to undiluted plasma, reduction in the number occurred gradually over a period of 96 hours. Thus, demonstrating that the maximum bactericidal effect depends upon the length of time that the plasma and bacteria are incubated together. Although bactericidal activity continues throughout 96 hours of incubation, the evaporation of the liquid is so great that it is difficult to interpret the results in terms of the original dilution. Therefore, a 48 hour incubation period was used in most of the experiments that follow.

II. Comparison of bactericidal activity of plasma and serum: Several tests were made to determine the bactericidal activity of plasma in comparison to that of serum. In every case plasma proved to be more active than serum. Table II illustrates the comparative differences when the procedure given in Experiment I, was used. According to these data, a 1:16 dilution of plasma has the equivalent activity of a 1:4 dilution of serum. Other comparative studies have

Table II

The bactericidal activity of bovine plasma as compared to that of serum.

Plasma or serum dilution	Plasma	Serum
	Colony counts after 10 hours incubation.	
1:2	0	0
1:4	0	10
1:8	0	46
1:16	10	Contaminated
1:32	28	50
1:64	36	1400
1:128	M	M
1:256	M	M
1:512	M	M
1:1024	M	M

No. of organisms added = 4×10^3.
Bacteria control after incubation = M.
M = Colonies too numerous to count.

been made on the bactericidal activity of plasma and serum and will be discussed in proceeding experiments.

III. Comparison of the bactericidal activity of plasma and whole blood: The procedure used in determining this difference is as follows: The bacterial dilutions were prepared as mentioned previously. A standard suspension of Br. abortus was diluted serially from 1:100 to 1:1,000,000. A uniform quantity (1 ml) of plasma or whole blood was added to each of a series of sterile vials in which had been placed 1 ml of the bacterial dilution. The contents were mixed, and then incubated at 37°C for 48 hours.

In Table III is set forth the differences between the bactericidal action of whole blood and that of plasma. Bovine whole blood was found to exhibit far less bactericidal power than plasma or serum. The method used in this experiment proved to be a more reliable measure of the bactericidal effect of blood than the one used in preceding experiments. Thus, this method was adopted as the standard procedure.

IV. Effect of time interval on the bactericidal activity of blood: It was noted during the progress of the study that the results on the plasma from the same animal often varied from day to day. Since no particular attention was paid to the time that elapsed between the collection of the blood from the animal and its examination, it was thought that the time interval might be an important factor in influencing the results.

Table III

Bactericidal action of plasma as compared with that of whole blood.

Number of bacteria added	Control after incubation	Plasma	Whole blood
	Colony count after 48 hours incubation		
1.2×10^3	M	0	2
1.2×10^4	M	0	400
6.0×10^4	M	0	1200
1.2×10^5	M	0	M
6.0×10^5	M	0	M
1.2×10^6	M	70	M
6.0×10^6	M	126	M
1.2×10^7	M	512	M

M = Colonies too numerous to count.

Table IV

The differences in the bactericidal activity of plasma held for different periods of time after collection.

Number of bacteria added	Control after incub.	Results of colony counts after 48 hours incubation															
		Plasma aged in presence of suspended cells								Plasma aged in presence of centrifuged cells							
		Interval between bleeding and examination															
		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	72 hrs.	192 hrs.	14 days	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	72 hrs.	192 hrs.	14 days
9.1×10^2	M	6	2	0	72	12	2	0	0	6	0	0	32	156	2	0	2
9.1×10^3	M	282	366	264	1246	1246	144	12	12	282	172	50	1246	3750	60	32	140
9.1×10^4	M	2500	5000	1800	2500	6250	768	262	100	2500	1220	420	M	M	776	368	5000
9.1×10^6	M	6250	7500	5500	M	M	3000	3000	1500	6250	6250	5000	M	M	8000	5000	M

M = Colonies too numerous to count.

A series of samples were collected from several animals and permitted to stand at different lengths of time and in different states before performing the tests. The results of one time interval experiment are shown in Table IV. It is obvious from the results that variations in bactericidal activity can be attributed to the time factor and also the state in which the plasma was held before its examination. The results of the examinations show that bovine plasma from different animals may show either an increase or decrease in bactericidal activity upon standing. Plasma separated from the blood cells or aged in the presence of cells was found to undergo a change in its activity. Similar experiments were conducted using serum in place of plasma. The same changes in activity were noted.

Numerous experiments were conducted to seek a plausible explanation for the changes which took place in the bactericidal activity of plasma when it remained for several hours before examination. Several test tubes containing plasma were divided into two groups. One group was plugged with cotton, and the other was sealed with rubber stoppers. Bactericidal tests were run at weekly intervals. The results showed that the plasma taken from the rubber stoppered tubes did not undergo the changes in bactericidal activity to the same extent as the plasma from the cotton plugged tubes. The tubes plugged with cotton would allow more aeration of the plasma. Thus, it was thought that by passing oxygen or carbon dioxide through the plasma one might attain the same alteration in bactericidal

activity that took place when the tubes of plasma remained for several hours at ice box temperature. Oxygen and carbon dioxide were passed separately through the samples of plasma, after which samples were sealed and placed at 4°C for 2 days. The treated samples were not significantly different in their bactericidal activity than the control plasma sample held for the same length of time.

Since the refrigeration of plasma samples does not prevent changes in their bactericidal activity, more accurate results might be expected if the samples are tested immediately after collection from the animal.

V. The effect of heat on bactericidal activity: Samples of plasma and serum were divided into three equal portions. One portion was heated at 56°C for one hour, another at 58°C for one hour, and the remaining left as a control. The method used in Experiment III was employed for comparing the bactericidal activity of these samples. Table V illustrates that heating at these mentioned temperatures markedly destroyed the bactericidal effect of both plasma and serum. However, even after heating, the activity of plasma was still greater than that of serum. The bactericidal activity of samples heated at 56°C, was not markedly different from that of the samples heated at 58°C.

VI. The effect of filtration on the bactericidal property of bovine plasma: To determine the effect of filtration upon the bactericidal property of bovine plasma, three different

Table V

The effect of heat on the bactericidal activity of bovine serum and plasma.

Number of bacteria added	Control after incub.	Plasma (unheated)	Serum (unheated)	Plasma 56°-1 hr.	Serum 56°-1 hr.	Plasma 58°-1 hr.	Serum 58°-1 hr.
		Colony count after 24 hours incubation					
1.4×10^3	M	4	84	128	538	158	540
1.4×10^4	M	80	960	1250	4130	1252	5000

M = Colonies too numerous to count.

Table VI

The effect of filtration on the bactericidal activity of bovine plasma.

Number of bacteria added	Control after incubation	Treatment of plasma			
		Unfiltered	Berkefeld	F. glass	Seitz
		Colony count after incubation			
8.75×10^{2}	M	0	1400	0	0
8.75×10^{3}	M	0	M	0	0
8.75×10^{4}	M	10	M	0	0
8.75×10^{6}	M	560	M	20	54

M = Colonies too numerous to count.

filters of different porosities were choosen, (1) a Berkefeld (W) which is known to retain complement, (2) a Seitz, and (3) a fine porosity Jena fritted glass filter. The filters were sterilized and the plasma drawn through into a sterile container by means of a vacuum pump.

The procedure used in determining the bactericidal property was the same as described in Experiment III. The results shown in Table VI brought forth a new aspect to the subject of bactericidins. While the Berkefeld filter completely removed all bactericidins, the more permeable Seitz and Jena fritted glass filter apparently retained an "antibactericidal" substance. As a result the bactericidal activity of the plasma was increased. The validity of these results were ascertained by repeating the procedure.

VII. The concentration of bactericidins: An effort was made to concentrate the bactericidins in bovine plasma by preforming the following experiment: Thirty mls of sterile bovine plasma were placed in a sterile cellophane tubing and dialyzed against C.P. glycerine; the sample being agitated constantly during the period of dialysis. The water was dialyzed rapidly out from the plasma, and within two hours the volume of plasma was reduced to 7.5 mls. Although highly concentrated, the syrup-like plasma remained in a perfectly clear state. The concentrated plasma, and an original sample were serially diluted and tested as in Experiment III. The results in Table VII show

Table VII

The effect of concentrating bovine plasma on bactericidal activity.

Plasma dilution	Plasma concentrated to 1/4 original volume	Normal plasma
	Colony count after incubation	
1:1	24	0
1:2	8	0
1:4	0	0
1:8	0	0
1:16	0	0
1:32	0	39
1:64	0	M
1:128	0	M
1:256	16	M
1:512	7800	M
1:1024	M	M

The number of bacteria added = 1000.
The number of bacteria after incubation = M.
M = Colonies too numerous to count.

that the highly viscous plasma was not as effective in killing the organisms in the first two dilutions as was the control plasma. However, the data indicate that it is more bactericidal in the higher dilutions.

VIII. The influence of the presence of Brucella agglutinins in plasma on bactericidal activity: In order to determine if any corrolation existed between the agglutination titer and bactericidal activity the following experiments were performed. A normal adult animal whose plasma showed no agglutinins in a 1:25 dilution or above was given an intravenous injection of a suspension of virulent Br. abortus organisms. The agglutination titer and bactericidal activity were recorded at weekly intervals for the first month after injection and thereafter at two week intervals. As shown in Table VIII, the bactericidal activity varied even when the agglutination titer remained constant. The bactericidal activity became progressively less in each succeeding test.

In a similar experiment conducted on a calf which had been vaccinated with a killed suspension of Br. suis. The rise and fall of the agglutination titer could in no way be corrolated with the flucuation taking place in the bactericidal activity of the plasma. The data on this animal is shown in Table IX.

X. The effect of the ingestion of colostrum by new born calves on bactericidins: The bactericidal activity of plasma from five different calves was determined before they ingested.

Table VIII

The relationship between the bactericidal activity and the agglutination titer of plasma of a cow following experimental injection.

Number of bacteria added	Control after incub.	Before vaccination	Agglutination titer after vaccination					
			7 days 1:2560	14 days 1:1280	21 days 1:1280	30 days 1:1280	47 days 1:640	54 days 1:640
	Colony count after 48 hours incubation							
8×10^2	M	0	0	0	4	4	108	140
8×10^3	M	2	12	20	430	350	3126	4000
8×10^4	M	6	208	340	M	2500	M	M
8×10^6	M	180	2600	4134	M	10500	M	M

M = Colonies too numerous to count.

Table IX

The relationship between the bactericidal activity and the agglutination titer of plasma from a calf following vaccination.

Number of bacteria added	Control after incub.	Before vaccination	Agglutination titer after vaccination			
			7 days 1:160	28 days 1:160	56 days 1:40	70 days 1:20
	Colony count after 24 hours incubation					
1.2×10^{5}	M	0	0	0	0	0
1.2×10^{5}	M	9	2	22	326	1500
1.2×10^{6}	M	284	24	232	M	M
1.2×10	M	1045	152	1000	M	M

M = Colonies too numerous to count.

colostrum and at various intervals up to several weeks afterwards. The results of this study on one animal are set forth in Table X, and are representive of all that were studied. The remarkable increase in bactericidal activity shortly after the ingestion of colostrum, and the progressive increase thereafter, was typical for all calves tested.

XI. Bactericidal activity of plasma of normal cows, those after recovery from infection and after vaccination: The results in Table XI are representative of a comparative study of the bactericidal activity of 15 normal cows, one adult cow known to have developed resistance to Brucella infection following recovery, 10 animals which were immunized by vaccination, 8 infected cows, and 3 cows that recovered from brucellosis infection as indicated by a marked decrease in their agglutination titers from a previous high level. The procedure used was the same as that of Experiment III. The number of bacteria added (as shown in Table XI) represents an average of the number of bacteria used in all the samples examined. All plasma samples examined from infected animals show little or no bactericidal power. Plasma of individual animals often vary considerably in bactericidal action but marked contrast between the colony counts from normal plasma-bacterial suspensions and those from infected animals is unusual and must have some significance. The activity of plasma from recovered animals was not equivalent to that of most normal animals. Little, if any difference was noted between the bactericidal action of normal, resistant, and vaccinated animals.

Table X

The effect of the ingestion of colostrum on the bactericidal activity of plasma from a new born calf.

Number of bacteria added	Control after incub.	Before ingestion of colostrum	3 days	1 month	2 months	3 months	4 months
	Results of colony counts after 48 hours incubation						
1×10^5	M	130*	0*	0	0	0	0
1×10^6	M	M*	40*	14	4	4	0
1×10^6	M	M*	170*	272	8	98	4
1×10^7	M	M*	556*	M	2000	3090	168

* = Incubated 24 hours.
M = Colonies too numerous to count.

Table XI

Comparison of the bactericidal action of plasma from a normal animal, resistant, vaccinated, infected animal, and one after recovery from the infection.

Number of bacteria added	Control after inoub.	Normal	Resistant	Vaccinated	Infected	Recovered
		Colony count after incubation				
1×10^3	M	0	0	0	1680	174
1×10^4	M	0	0	0	M	5000
1×10^5	M	2	2	3	M	M
1×10^6	M	48	40	34	M	M
1×10^7	M	690	708	734	M	M

M = Colonies too numerous to count.

XII. The comparative susceptibility of the three species of Brucella to bactericidal action: One smooth strain of each of the species of Brucella was selected for the purpose of demonstrating their comparative susceptibility to the bactericidins in bovine plasma. The bacterial suspensions were prepared in the usual manner. The plasma was that of an normal animal and the procedure described in Experiment III was used. The results are tabulated in Table XII. Br. suis was found to be the most resistant of the three species to the action of bactericidins, while Br. abortus was the most suspectible.

XIII. The comparative susceptibility of rough (R) and smooth (S) types of Br. abortus to the bactericidal action of bovine plasma: A smooth culture of Br. abortus was dissociated by multiple transfers in sterile broth consisting of 1.0 per cent Tryptose peptone, 3.0 per cent dextrose, and 0.5 per cent NaCl. Standard suspensions of the R and S types were prepared as previously described. The plasma samples were from a normal, vaccinated, and infected cow. The procedure described in Experiment III was used. The data are tabulated in Table XIII.

The results show that the rough type of organism is far more susceptible to the bactericidal action of bovine plasma than is the smooth type. The contrast is especially pronounced in the plasma from the infected cow. As shown previously, plasma from infected animals has relatively no bactericidal activity towards

Table XII

The comparative susceptibility of the three species of Brucella to the bactericidal action of bovine plasma.

Species	Number of bacteria added	Control after incubation	Plasma-bacterial suspension
		Colony count after incubation	
Brucella abortus	7×10^2	M	0
	7×10^3	M	6
	7×10^4	M	138
	7×10^6	M	1442
Brucella suis	1×10^5	M	618
	1×10^5	M	M
	1×10^6	M	M
	1×10^7	M	M
Brucella melitensis	7×10^2	M	10
	7×10^3	M	M
	7×10^4	M	M
	7×10^6	M	M

M = Colonies too numerous to count.

Table XIII

The comparative susceptibility of rough and smooth Br. abortus to the bactericidal activity of plasma from a normal, vaccinated, and infected cow.

Number of bacteria added	Control after incubation	Normal animal	Vaccinated animal	Infected animal
		Colony count after 48 hours incubation		
S 1.1 x 10^8	M	0	0	16
R 1.2 x 10^8	M	0	0	0
S 1.1 x 10^6	M	0	0	M
R 1.2 x 10^6	M	0	0	0
S 1.1 x 10^6	M	6	4	M
R 1.2 x 10^6	M	0	0	0
S 1.1 x 10^7	M	20	20	M
R 1.2 x 10^7	M	2	10	6

S = Smooth organisms.
R = Rough organisms.
M = Colonies too numerous to count.

smooth strains of Br. abortus. In the case of R type, the plasma is as bactericidal as plasma from a normal or vaccinated animal. In view of the differences in susceptibility to bactericidins shown by a rough strain, it would seem important to use a culture in a bactericidal system that is free from dissociated cells..

DISCUSSION

Topley and Wilson (15) define complement as ---" a thermolabile substance, or complex of substances, present in varying concentrations of blood serum of most normal animals, which has the property of bringing about lysis of certain cells and bacteria in conjunction with certain antibodies which render the cells or bacteria sensitive to its action." They consider their definition incomplete because it is still an open question as to the exact role of complement and antibody in the anti-bacterial property of normal serum. Many theories have been proposed to explain the combined activity of "bacteriophilic complement" and "specific antibody." In 1909 Muir and Browning (8) began a series of experiments designed to determine whether or not complement could be absorbed out by homologous bacteria and thereby diminish the bactericidal activity on both homologous and heterogenous organisms. They found that the treatment of a normal serum with increasing amounts of a killed suspension of a bacterium, usually produced effects in the following order: (a) diminution of the bactericidal action on the same bacterium; (b) diminution of the bactericidal action on other bacterium; (c) diminution of hemolytic complement. The latter effect brings forth an important question: "Are bacteriophilic

complement and hemolytic complement analogous?" A corrolary existing between the findings of Noguchi and Bronfenbrenner (10) and the data tabulated in Table IV, may be of significance. In their study of the variation in complement activity of guinea pigs, they noted that a large number of serums gained in complementary activity when remaining in contact with the clot for 46 hours, while some serums become weakened during the same length of time. Their observation coincides with the finding that the bactericidal activity of plasma or serum may increase or weaken upon standing. The interval of time elapsing between the withdrawal of the blood and the testing of its activity has proved to have an important bearing on the quantitative measurement of bactericidins. Of further interest is the fact that plasma or serum does not have to be in contact with blood cells or clot in order to accomplish a rise in bactericidal power. Therefore, one can exclude the possibility of leococytic extracts (leukins), or other cellular components, as being responsible for the increase in activity. Upon standing, either a substance which inhibits bactericidal activity is diminished, or a bactericidal agent is set free from a plasma constituent. If the increase in bactericidal activity was found to be proportional to the increase in complement activity, one could say that "hemolytic complement" and "bacteriophilic complement" were analogus. However, even though the supporting evidence is a prologue to this theory, it would require further investigation before such a conclusion could be reached. In contrast to this proposal, Dunlop (1) claims that unlike hemolytic complement, the complement concerned in

bactericidal action is absorbed by charcoal. It is generally supposed that there are more than one component involved in the bactericidal phenomenon. Therefore it would not necessarily have to be the complement absorbed by charcoal, but possibly the so-called "sensitizing antibody."

The length of time that blood-bacterial suspensions are incubated together is of importance in determining the anti-bacterial effect of whole blood, serum, or plasma. In low dilutions of plasma (1:2 to 1:16) the killing of Brucella organisms is a gradual process, and the diminition of bacteria is directly proportional to the time of incubation. In higher dilutions of plasma (1:32 and above), the number of organisms shows a gradual decline for the first few hours and then a sudden rise upon further incubation. One explanation of this may be the proportion of antibody to antigen which is not sufficient to inhibit the growth over an extended period of time. When antibody is present in sufficient quantity to sensitize all the bacteria to the lytic action of complement, the decline in bacteria depends upon the incubation period. By using a constant amount of plasma, and varying the number of bacteria, the degree of killing obtained depends on the proportion of bacteria to bactericidins and the incubation period. For comparative purposes, a 48 hour incubation period proved satisfactory for the species of Brucella.

The incubation period for in vitro bactericidal tests is dependent upon the bacterium employed. In an experiment by Mackie and Finkelstein (7) it was noted that after an incubation

period of three hours, for E. typhosa and certain other organisms, maximum effect was obtained and further incubation did not elicit appreciably greater killing.

No explanation can be given for the greater bactericidal activity displayed by plasma in comparison with serum. The only known difference between plasma and serum is the presence of fibrinogen in the former. Yet fibrinogen, when separated from plasma, displays no bactericidal effect. Fibrinogen is precipitated when plasma is heated at 56°C for one hour. Plasma retains more bactericidal activity than does serum when treated at the same temperature. This is an indication that a bactericidal agent exists in plasma which is not present in serum.

Irwin and associates (5) compared the bactericidal activity of a plasma cell mixture with that of whole blood. Their findings were that the activity of this mixture was never more pronounced, and occasionally less than that of whole blood. However, the blood cells present in the plasma may have lowered its activity. Citrated whole blood has little, if any bactericidal action against Br. abortus. The difference in the activity of plasma and whole blood is especially pronounced in normal animals. Where complete killing of 1200 organisms by 1 ml of whole blood has not been noted, 1 ml of plasma from the same animal may kill as high as 60,000 Br. abortus organisms. This finding is representative of the difficulties encountered when trying to correlate in vitro results with the bactericidal phenomenon that occurs in vivo.

It has been recognized generally that hemolytic and bacteriophilic complement are destroyed by heating normal serum at 55°C for 30 min.

The bacteriophilic complement is considered by Mackie and Finkelstein (6) to be the active principle in the killing of Gram-negative organisms, and when it is destroyed by heating the bactericidal activity is lost. They assert that Gram-positive organisms are acted upon by a separate mechanism, because of the thermostability of the bactericidal agent. The occurrence of heat stable bactericidal properties in serum has also been studied by Selter (13), who believes that different organisms are affected by a common agent and not by multiple specific agents. Although the bactericidal activities of both bovine plasma and serum were markedly weakened by heating at 56°C for 1 hour, it was noted that their activity towards Br. abortus was not totally destroyed. The thermostable bactericidins, as suggested by Selter, may be a common agent to both Gram-negative and Gram-positive bacteria, while the labile complement may be needed as a specific agent in killing Gram-negative organisms.

It has long been known that hemolytic complement could be removed from serum by means of a filter of exceedingly fine porosity. The Berkefeld filter has proved most satisfactory. By filtration through a Berkefeld (W), plasma looses all of its bactericidal properties. If one attributes this loss to bacteriophilic complement retention, then this again brings it in close relationship to hemolytic complement. In comparing the activity of filtrates from Berkefeld, Seitz, and Jena fritted glass filters, a very interesting resultant was noted. While Berkefeld filters deprived the plasma of its activity, the Seitz and fritted

glass increased the bactericidal effect. This increase may be explained by the retention of either an anticomplementary substance or an inhibitor of bactericidins. In either case the size of this substance must be larger than that of the bactericidins which passed through.

No appreciable increase in bactericidal activity can be obtained by concentrating plasma. The change in viscosity, or the altering of the combining action of the bactericidins in a high concentrated plasma appears to retard bactericidal action. By diluting the plasma back up to its original volume, it regains its original activity.

Gengou (4) in 1899 drew attention to the fact that bacteria are agglutinated as well as lysed by a specific antiserum and that this effect may greatly reduce the number of colonies in a plate count; the clumping of the bacteria producing single colonies. For this reason, Mackie and Finkelstein (7) assert that bactericidal tests which have been based on colony counts give an unreliable index of bactericidal activity. It is acknowledged here that the agglutination titer of plasma may have an indirect bearing on the number of colonies counted. However, as a comparative index of the bactericidal activity of plasma from normal or animals showing high agglutination titers, this method has proved very reliable. As shown in Table VIII and IX, when the agglutinins are not demonstrable in plasma in a dilution of 1:25 or above the bactericidal action of the plasma is markedly higher before the same animal has developed specific agglutinins. If the bacteria placed in the presence of a high titered plasma were agglutinated, a lower colony count should be obtained than when the plasma was negative. When plasma samples from a single animal show

a constant high agglutination titer over a period of weeks, the colony counts in a standard bactericidal test should remain approximately the same, if the titer influenced the resultant bacterial counts. This was not observed.

If the resistance of an animal to brucellosis was in direct relationship to the bactericidal action of its plasma towards Br. abortus in vitro, then upon immunization of the animal an increase in bactericidal activity should occur. However, the reverse is often the case. Calves which were immunized by vaccination, exhibited more bactericidal activity before immunization then after. The bactericidal antibodies are undoubtedly of different character then those responsible for resistance to infection. As yet, bactericidal tests have not proved to be a measure of immunity or susceptibility of an animal to any specific infection.

The plasma from new born calves, before they have ingested colostrum, has little, or no bactericidal effect. Forty-eight hours after the ingestion of colostrum there is a sudden increase in bactericidal action, and continues to rise for several weeks. The age at which calves show maximum bactericidal activity cannot be definitely stated, as it varies with the animal. In certain animals studied, the bactericidal activity of the plasma from calves 3 days old were within the range of that of an adult cow. As a collorary to this study, it is of interest to cite the findings of San Clemente and Huddleson (12) in --- "Electrophoretic studies of the proteins of bovine serums with respect to Brucella." From the electrophoretic studies upon the serum of a calf at birth,

before the ingestion of colostrum, it was observed that, (a) calf serum obtained before the ingestion of colostrum showed a high concentration of α -globulin which might almost equal or even exceed the concentration of albumin; (b) γ -globulin was extremely low or absent. Six days after birth and after it had ingested colostrum the following changes had taken place: (a) the high α peak had decreased from 44.2 to 30.4 per cent of the total area, while the γ peak had risen from 2.3 to 20.7 per cent. Twenty-two days later the electrophoretic pattern began to approach that of an adult cow. As γ -globulin is associated with the antibody content of serum, one might assume that the sudden rise in γ -globulin was responsible for the rise in bactericidal activity. However, the fact that infected cow's serum has a much higher γ -globulin content then does that of an normal animal, but a lower bactericidal capacity, would tend to disprove any such assumption. Nevertheless, colostrum either provides bactericidins or stimulatesthe production of these agents.

The bactericidal property of calf plasma is developed at an early age. Within three days after birth the bactericidal capacity may have reached the extent of an adult animal, or even exceed it. It is a well established fact that calves are highly resistant to Brucella infection. Whether high resistance and high bactericidal activity on the part of plasma are related in any way is problematical. Since plasma from adult animals that have never knowingly been exposed to Brucella infection, in many instances, show just as high bactericidal activity it would appear

that such a question cannot be answered satisfactorily until such adult animals have been exposed to infection to determine their susceptibility.

The changes in the bactericidal activity of bovine plasma cannot be correlated with the age of the animal. Occasionally, plasma from a normal adult animal may have greater bactericidal activity than that of an month old calf. It is evident that the factors governing the bactericidins in vivo are multiple. The diet of the animal may play a part in this role. Ecker and associates (2) have shown that a correlation exists between the concentration of ascorbic acid in the blood serum of guinea pigs and the complementary activity of the serum. It is possible that a relationship may also exist between ascorbic acid and bactericidal activity, as complement undoubtedly plays a part in the bactericidal mechanism.

The antibacterial activity of plasma from acquired immune, and animals immunized by vaccination lies within the range of the variable activity of that of normal animals. Although immune individuals may be resistant to Brucella infection, the resistance cannot be foretold by means of a bactericidal system. The absence of bactericidal activity in plasma from infected animals, and the low activity of plasma from those having recovered from infection must have immunological significance. Fiessinger and Cattan (3) report a disappearance of the bactericidal action of whole blood in typhoid fever patients, a phenomenon similar to that noted in bovine plasma from infected animals. Irwin

and associates (5) had likewise observed that certain cows, recovered from a previous infection of Br. abortus, showed a loss in bactericidal properties of whole blood as compared to that of presumably normal animals.

Striedter and Avrukina (14) were unable to demonstrate any differences in the bactericidal activity of serums from healthy guinea pigs (highly susceptible) or those infected with or recovered from brucellosis. In view of these results, the differences noted in the bactericidal activity of plasma from normal and infected cows may not hold true for guinea pigs.

It has been generally assumed that a sensitizing antibody takes part in bactericidal phenomena. Mackie and Finkelstein (7) regard antibacterial antibodies as being highly specific. These specific antibodies, if related to antibodies produced by immunization, should be greatly increased during infection. However, the antibacterial effect is lessened. If an optimum proportion of combining power exists between antibody and complement, or antibody and antigen to obtain bactericidal activity, then an increase in antibody content may decrease the action of complement. It was noted by Neisser and Wecksberg (9) that a marked pro-zone often occured in bactericidal tests. A particular dilution of serum might exert no bactericidal action, while a much higher dilution resulted in the complete killing of the bacteria. In accordance with these findings, a pro-zone phenomenon was observed whenever extremely high dilutions

of plasma were tested for bactericidal effect upon a constant number of organisms. The high activity shown in the pro-zones may be due to the existance of the proper ratio of antibody to antigen and not the proportion of complement to antibody. If the pro-zone of bactericidal action depends upon the presence of an optimum amount of antigen to antibody then by serial dilution one may arrive at a dilution in which the optimum combining proportion obtains for bactericidal activity. However, since it was found in this study that more than one pro-zone occurred, this would hardly serve as an explanation.

The literature is incomplete on the susceptibility of the species of Brucella to bactericidal action of bovine plasma. Irwin and associates (5) have found Br. suis to be more resistant to the bactericidins of bovine blood than Br. abortus. While Striedter and Avrukina (14) have noted Br. melitensis relatively more resistant to the bactericidins in serums of guinea-pigs and sheep than Br. abortus. The arrangement of the three species in regards to their reactivity to normal bovine plasma is as follows: (a) Br. abortus is markedly more susceptible than the other two; (b) Br. melitensis is less resistant than Br. suis.

The rough type of Br. abortus is far more susceptible to the bactericidal activity of bovine plasma than the smooth type. Plasma from an infected animal is as highly bactericidal to the rough type as that of a normal animal. In accordance with these findings is the work of Mackie and Finkelstein (6).

They noted the R variant of E typhosa proved more susceptible to serum bacteriolysis than the S form.

SUMMARY

1. The interval of time elapsing between the withdrawal of blood from the animal and the testing of its bactericidal activity has a direct bearing on the bactericidal action of whole blood, serum, or plasma. The majority of plasma samples increased in activity upon standing at 4°C for 48 hours or more, while a few samples diminished in activity. Because of this unpredictable change in activity it was found that more accurate results could be obtained when the samples were examined immediately after withdrawal from the animals.

2. The bactericidal action of bovine plasma in vitro is not one of abrupt killing, but a gradual process. The effectiveness of this action is dependent upon the time of incubation, and the ratio of plasma to the number of organisms used. As a comparative method, a constant amount of plasma added to varying numbers of organisms proved more satisfactory.

3. Plasma has a greater bactericidal action than does serum. Whole blood shows only a slight bactericidal activity.

4. The activity of plasma and serum is markedly lowered by heating at 56°-58°C for one hour but is not totally destroyed. Fibrinogen is precipitated at this temperature, but plasma still retains a higher activity than does heated serum. Therefore, the difference in bactericidal activity of serum and plasma cannot be

accounted for by the presence of fibrinogen.

5. The bactericidal property of plasma can be removed by filtration through a Berkefeld (W). The more porous filters such as Seitz and Jena fritted glass retained an "antibactericidal" substance, and thus the filtrates show a greater bactericidal effect than does the unfiltered control sample.

6. There was an appreciable loss in bactericidal activity when plasma was concentrated to a highly viscous state. The activity was regained upon diluting the concentrate back to its original volume.

7. The agglutination titer of bovine plasma cannot be corralated with its bactericidal activity. The presence of agglutinins in the plasma proved incidental to the comparative index of the bactericidal activity of normal plasma to those which showed high titers.

8. Plasma of new born calves, before they have ingested colostrum, has little, or no bactericidal activity. Shortly after the ingestion of colostrum the activity increases and become progressively greater as the calf matures.

9. Plasma from cows infected with brucellosis has little or no bactericidal effect upon Br. abortus. Plasma from animals that have recovered from infection show a lower bactericidal activity than do normal animals. No apparent differences were shown in the bactericidal activity of normal animals, acquired immune, and vaccinated cows.

10. The species of Brucella vary in their susceptibility to the action of bactericidins in bovine plasma; Br. abortus being the most susceptible, Br. melitensis and Br. suis the least.

11. The rough type of Br. abortus is far more susceptible to the bactericidal action of bovine plasma than is the smooth type.

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