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Further Studies of the Test-Tube Agglutination Test for the Diagnosis of Bang's Disease (Contagious Abortion)

C. P. Fitch, C. R. Donham, and W. L. Boyd
Division of Veterinary Medicine

UNIVERSITY FARM, ST. PAUL

CONTENTS

FURTHER STUDIES OF THE TEST TUBE AGGLUTINATION TEST FOR THE DIAGNOSIS OF BANG'S DISEASE (CONTAGIOUS ABORTION)[1]

C. P. FITCH, C. R. DONHAM, and W. L. BOYD

INTRODUCTION

This is the second publication on the agglutination test for the diagnosis of Bang's disease. The first eleven articles were included in Technical Bulletin No. 73, of the University of Minnesota Agricultural Experiment Station. These studies were made possible by a grant from the Bureau of Animal Industry, United States Department of Agriculture.

XII. EFFECT OF VARIATIONS IN HYDROGEN-ION CONCENTRATION OF *BACT. ABORTUS* ANTIGENS ON THE AGGLUTINATION REACTIONS OF BOVINE SERA TESTED WITH SUCH ANTIGENS

Many laboratories do not determine the H-ion concentration of *Bact. abortus* antigen used in the test tube agglutination test for the diagnosis of Bang's disease in animals or undulant fever in man. Some laboratories establish the H-ion concentration of such antigens at or near the neutral point. The H-ion concentration of the culture medium (nutrient agar) on which the bacteria are grown for the production of the antigen is usually near the neutral point. It is generally recognized that some change in the H-ion concentration of the antigen frequently takes place unless an adequate buffer is present in the material.

The object of these studies was to determine the influence of variations in H-ion concentration of *Bact. abortus* antigens on the results of the test tube agglutination test.

We failed to find in the literature any reports of studies of the effect of variations in H-ion concentration of *Bact. abortus* antigens on their agglutinability.

Acid agglutination and salt agglutination of micro-organisms and the characteristics and action of agglutinins are discussed by Buchanan and Fulmer (1). In a discussion of the mechanism of specific agglutination they say: "Apparently the action of agglutinin is specifically to sensitize the homologous antigen (cell) to the action of electrolytes . . . Many suggestions have been made as to the mechanism of agglutination. Apparently all may be discarded except those which emphasize the colloidal character of the reaction."

Northrop and de Kruif (2) studied agglutination of suspensions of the typhoid bacillus. It was found that the amount of agglutinin com-

bined with the cells "is quite constant from pH = 9 to pH = 3.7." Below pH = 3.7 the amount decreases. The addition of immune serum to a suspension of typhoid bacilli at pH = 2.5 increased the positive charge of the cells. They conclude that: "This quite contradicts the concept that union of antigen and antibody is due to differences in the sign of their charges, that the agglutinin forms a film over the surface of the organism, and that the effect on the charge is the result of this film. Apparently the amount of agglutinin taken up by the cell and required for agglutination depends in part upon the concentration of the salt."

Buchanan and Fulmer (3) state that: "In general there is a broad zone of pH within which the rates of growth are quite uniform for those short periods during which the increase in viable cells approaches the logarithmic rate. On the borders of these zones of pH slight change in the pH produces a marked effect upon reproduction." Also data are given for a list of micro-organisms showing the maximum, minimum, and optimum pH values for the growth of bacteria, but *Bact. abortus* is not included. These factors "are not fixed, but are dependent upon other environmental conditions. Different acids of the same pH do not exert the same influence in inhibiting cell multiplication."

Lisse (4) reports that: "Attention has been paid to the effect of the addition of electrolytes to the medium used for growth of bacteria on the charge carried by such bacteria. A change in the initial pH of the growth medium (nutrient agar) from pH 5.0 to 8.8 does not bring about a change in charge of the organisms obtained from them."

Tittsler and Lisse (5) in reporting studies with *Salmonella pullorum* antigens state: "A relationship between electrophoresis, agglutinability and virulence is suggested."

Evans (6) proposed a practical method of producing a buffered physiological salt solution for use in serological tests. She says: "The influence of variation in H-ion concentration of the medium has not been determined for certain serological reactions used commonly for test purposes, but there is a general recognition of a relationship between H-ion concentration and agglutination." She conducted agglutination tests with suspensions of meningococci and immune rabbit serum. The H-ion concentration of the salt solution used to suspend the bacteria was varied from pH 5.6 to 8.6. Agglutination titres of sera were higher with the bacterial suspensions that were slightly acid.

Coulter (7) studied the iso-electric point of red blood cells and its relation to agglutination. He gives the following conclusions: "The movement of normal and sensitized red blood cells in the electric field is a function of the hydrogen ion concentration. The iso-electric point, at which no movement occurs, corresponds with pH 4.6. On the alkaline side of the iso-electric point the charge carried is negative and

increases with the alkalinity. On the acid side the charge is positive and increases with the acidity."

Mathews (8) reported that: "The agglutination test for bacillary white diarrhea is frequently obscured by a precipitate, the principle constituent of which is protein. Two cubic centimeters of a 2 per cent water solution of NaOH added to 100 cc. of *E. sanguinaria* antigen did not influence its agglutinability and eliminated 95 per cent of the 'cloudy tests' when added just before setting up the test."

Ecker and Simon (9) studied acid agglutination optimum in the Brucella group of organisms as a means of differentiating strains. They state: "It is therefore evident that all the strains used completely agglutinate at the same pH level, namely, from 3.18 to 2.14."

Huddleson (10) recommends that the culture medium (liver infusion agar) be adjusted to have a pH of 6.4 to 6.8 for isolating and growing *Bact. abortus*. He states: "The growth of *Bact. abortus* in culture is markedly influenced by the H-ion concentration of the medium. It is important that the medium be adjusted in terms of H-ion concentration."

In our work we have employed polyvalent *Bact. abortus* antigens, prepared by washing the bacteria from agar slant cultures in physiological saline solution containing 0.5% phenol as a preservative. The concentration of the antigen was adjusted to 0.04% bacteria by the centrifuge tube method (11). Subsequently the stock antigen was divided into several flasks and various amounts of dilute solutions of hydrochloric acid and sodium hydroxide were added to the antigens. Fourteen antigens with different concentrations of HCl and nineteen antigens with different concentrations of NaOH were used. The pH concentration values of the antigens were determined by electrometric and colorometric methods. Agglutination tests were conducted with these antigens. Six bovine sera (three high agglutinin content, titre 1:500 or above; two low to medium agglutinin content, titre 1:25 to 1:100; and one with no agglutinin) were tested with the antigens containing various concentrations of HCl and fifteen bovine sera (nine high agglutinin content, five low to medium agglutinin content, and one no agglutinin content) were used with the antigens containing various concentrations of NaOH.

The technic of preparing the serum-antigen dilutions was the same as described in a previous publication (11). Maximum titres were determined for high agglutinin content sera with all of the different antigens. Stock polyvalent antigen was used as a check on the maximum titres of all agglutinating sera. The agglutination tests were held in a 37.5° C. incubator and observations made at 24, 48, and 72 hours to record the rate of agglutination and the maximum titres attained by the agglutinating sera.

Two series of antigens containing HCl were used and the experiment was repeated three times with antigens containing NaOH.

The pH value of the stock antigen was approximately 6.9 to 7.1. Antigens to which HCl was added showed a range of pH values extending from approximately 7.1 to 2.9; those in which NaOH was added showed pH values from 7.1 to 9.6.

Table 1

Typical Results of Agglutination Tests with Bact. abortus Antigens Containing Various Concentrations of HCl

Dilutions: 1-25, 1-50, 1-100, 1-250, 1-500, 1-1000
72-hour observation

pH Concentration of antigen	Serum 130						Serum 533						Serum 129						Serum 413					
Stock																								
7.168	+	+	I	—	—	—	+	+	+	+	+	I	+	+	+	—	—	—	+	+	+	+	+	+
7.117	+	+	I	—	—	—	+	+	+	+	+	I	+	+	I	—	—	—	+	+	+	+	+	I
7.100	+	+	I	—	—	—	+	+	+	+	+	I	+	+	I	—	—	—	+	+	+	+	+	+
7.050	+	+	+	—	—	—	+	+	+	+	+	I	+	+	I	—	—	—	+	+	+	+	+	+
6.881	+	+	+	—	—	—	+	+	+	+	+	—	+	+	—	—	—	—	+	+	+	+	+	I
6.847	+	+	+	—	—	—	+	+	+	+	+	I	+	+	I	—	—	—	I	I	+	+	+	+
6.204	+	+	I	—	—	—	+	+	+	+	+	I	+	+	I	—	—	—	+	+	+	+	+	+
4.818	+	+	+	I	—	—	+	+	+	+	+	I	+	+	I	—	—	—	+	+	+	+	+	+
4.700	+	+	I	—	—	—	+	+	+	+	+	I	+	+	I	—	—	—	+	+	+	+	+	—
											A	A					A	A					A	A
3.584	+	I	—	—	—	—	+	+	+	—	+	+	+	I	—	—	+	+	+	+	+	—	+	+
				A	A	A				A	A	A			A	A	A	A					A	A
3.483	+	+	I	+	+	+	+	+	+	I	+	+	+	+	I	+	+	+	+	+	+	—	+	+
				A	A	A				A	A	A				A	A	A					A	A
3.449	+	I	—	I	+	+	+	+	I	+	+	+	+	—	—	+	+	+	I	+	+	—	+	+
			A	A	A	A				A	A	A				A	A	A			A	A	A	A
3.212	+	I	+	+	+	+	+	+	—	+	+	+	I	—	—	+	+	+	+	+	I	+	+	+
			A	A	A	A				A	A	A			A	A	A	A		A	A	A	A	A
2.908	+	I	+	+	+	+	+	—	—	+	+	+	I	—	+	+	+	+	+	I	I	+	+	+

pH Concentration of antigen	Serum 21						Serum 86											
	1–25	1–50	1–100	1–250	1–500	1–1000	1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000	1–6000	1–7000
Stock																		
7.168	—	—	—	—	—	—	+	+	+	+	+	+	+	+	—	+	—	—
7.117	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	—	—
7.100	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	I	—	—
7.050	—	—	—	—	—	—	+	+	+	+	+	+	+	+	I	I	—	—
6.881	—	—	—	—	—	—	+	+	+	+	+	+	+	I	I	I	—	—
6.847	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	I	—	—
6.204	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	—	—	—
4.818	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	—	—
4.700	—	—	—	—	—	—	+	+	+	+	+	+	+	—	—	—	—	—
					A	A					A	A	A	A	A	A	A	A
3.584	—	—	—	—	+	+	+	+	+	I	+	+	+	+	+	+	+	+
				A	A	A			A	A	A	A	A	A	A	A	A	A
3.483	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
				A	A	A			A	A	A	A	A	A	A	A	A	A
3.449	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			A	A	A	A			A	A	A	A	A	A	A	A	A	A
3.212	—	—	I	+	+	+	+	+	I	+	+	+	+	+	+	+	+	+
		A	A	A	A	A		A	A	A	A	A	A	A	A	A	A	A
2.908	—	+	+	+	+	+	+	I	+	+	+	+	+	+	+	+	+	+

+ = Complete agglutination. — = No agglutination.
I = Incomplete agglutination. A = Acid agglutination.

Table 2

Typical Results of Agglutination Tests with Bact. Abortus Antigens Containing Various Concentrations of NaOH

pH Concentration of antigen	Dilutions: 1–25, 1–50, 1–100, 1–250, 1–500, 1–1000, 1–2000, 1–3000, 1–4000, 1–5000, 1–6000		
	Serum 86	Serum 153	Serum 143
Stock 6.999	+ + + + + + + + +	+ + + + + + + + I – –	+ + + + + + + + I I –
7.151	+ + + + + + + + I I	+ + + + + + + I I – –	+ + + + + + + I – – –
7.286	+ + + + + + + + + I	+ + + + + + + + – – –	+ + + + + + + I – – –
7.557	+ + + + + + + + + –	+ + + + + + + I – – –	+ + + + + + + I – – –
7.743	+ + + + + + + + – –	+ + + + + + + I – – –	+ + + + + + + – – – –
7.810	+ + + + + + + +	+ + + + + + + – – – –	+ + + + + + + – – – –
7.929	+ + + + + + + + – –	+ + + + + + – – – – –	+ + + + + + + I – – –
8.013	+ + + + + + + + – –	+ + + + + + + I – – –	+ + + + + + + + – – –
8.081	+ + + + + + + + + +	+ + + + + + + I – – –	+ + + + + + + I – – –
8.233	+ + + + + + + + I –	+ + + + + + + I – – –	+ + + + + + + – – – –
8.318	+ + + + + + + + I –	+ + + + + + + I – – –	+ + + + + + + I – – –
8.487	+ + + + + + + + I I	+ + + + + + I + – – –	+ + + + + + + + – – –
8.656	+ + + + + + + + + –	+ + + + + + + + – – –	+ + + + + + + – – – –
8.876	+ + + + + + + + I I –	+ + + + + + + I – – –	+ + + + + + + I – – –
8.994	+ + + + + + + + I I	+ + + + + – I – – –	+ + + + + + + + – – –
	S S S S	S S S	S S S
9.146	+ + + + + + + + + –	+ + + + + + + – – – –	+ + + + + + I – – – –
	S S S S	S S S S	S S S
9.281	+ + + + + + + + + –	+ + + + + + + + – – –	+ + + + + + I – – – –
	S S S S	S S S S	S S S S S
9.332	+ + + + + + + + I –	+ + + + + + + – – – –	+ + + + + + + I I – –
	S S S S S	S S S S S S	S S S S S
9.417	+ + + + + + + + I –	+ + + + + + + + – – –	+ + + + + + + I I – –
	S S S S S S	S S S S S S	S S S S S
9.636	+ + + + + + + + – –	+ + + + + + + I – – –	+ + + + + + + + + – –

+ = Complete agglutination. – = No agglutination.
I = Incomplete agglutination. S = Stringy agglutination.

Table 2—Continued

Typical Results of Agglutination Tests with Bact. abortus Antigens Containing Various Concentrations of NaOH

Dilutions: 1-25, 1-50, 1-100, 1-250, 1-500, 1-1000

pH Concentration of antigen	Serum 533	Serum 413	Serum 189
Stock 6.999	+ + + + + −	+ + + + + +	+ + + + + +
7.151	+ + + + + −	+ + + + + I	+ + + + + +
7.286	+ + + + + −	+ + + + + I	+ + + + + +
7.557	+ + + + + −	+ + + + + I	+ + + + + +
7.543	+ + + + + −	+ + + + + −	+ + + + + I
7.810	+ + + + + −	+ + + + + I	+ + + + + +
7.929	+ + + + + −	+ + + + + I	+ + + + + +
8.013	+ + + + + −	+ + + + + I	+ + + + + +
8.081	+ + + + + −	+ + + + + +	+ + + + + I
8.233	+ + + + + −	+ + + + + I	+ + + + + +
8.318	+ + + + + −	+ + + + + I	+ + + + + +
8.487	+ + + + I −	+ + + + + −	+ + + + + +
8.656	+ + + + + −	+ + + + + I	+ + + + + I
8.876	+ + + + + −	+ + + + + I	+ + + + + +
8.994	+ + + + + −	+ + + + + +	+ + + + + I
	S S S S	S S S	S S S S S S S
9.146	+ + + + + I	+ + + + − −	+ + + + + + + I
	S S S	S S S S	S S S S S S
9.281	+ + + + + I	+ + + + + −	+ + + + + I I
	S S S	S S S S	S S S S S S
9.332	+ + + + + I	+ + + + + −	+ + + + + + − −
	S S S S	S S S	S S S S S
9.417	+ + + + + +	+ + + + + −	+ + + + + + − −
	S S S S S	S S S S S	S S S S S
9.636	+ + + + + +	+ + + + + I	+ + + + + + I −

pH Concentration of antigen	Serum 129	Serum 130	Serum 138
Stock 6.999	+ + I − − −	+ + − − − −	+ + I − − −
7.151	+ + − − − −	+ + − − − −	+ + + − − −
7.286	+ + I − − −	+ + − − − −	+ + I − − −
7.557	+ + − − − −	+ + − − − −	+ + I − − −
7.743	+ + I − − −	+ + − − − −	+ + + − − −
7.810	+ + I − − −	+ + − − − −	+ + + − − −
7.929	+ + I − − −	+ + − − − −	+ + I − − −
8.013	+ + I − − −	+ + − − − −	+ + + − − −
8.081	+ + I − − −	+ + − − − −	+ + + − − −
8.233	+ + I − − −	+ + − − − −	+ + I − − −
8.318	+ + I − − −	+ + − − − −	+ + + − − −
8.487	+ + I − − −	+ + − − − −	+ + + − − −
8.656	+ + I − − −	+ + − − − −	+ + + − − −
8.876	+ + I − − −	+ + I − − −	+ + I − − −
8.994	+ + I − − −	+ + − − − −	+ + + − − −
		S	
9.146	+ + − − − −	+ I − − − −	
		S S	
9.281	+ + I − − −	+ + I − − −	
		S	
9.332	+ + I − − −	+ + − − − −	
		S S	
9.417	+ + I − − −	+ + − − − −	
	S	S S	
9.636	+ + + − − −	+ + − − − −	

+ = Complete agglutination. − = No agglutination.
I = Incomplete agglutination. S = Stringy.

Results

The agglutination reactions of sera with a wide range of agglutinin content were not appreciably influenced when tested with antigens of pH values between 4.7 and 8.9. The slight discrepancies recorded for the titres of agglutinating sera tested with antigens having pH values in the above zone were within the limits of experimental error and were not significant.

The titres of agglutinating sera were markedly influenced when the antigens with which they were tested contained sufficient acid to result in pH values of approximately 3.5 or less. There was a definite inhibition of the agglutination reaction in such antigens. Also, acid agglutination was observed in the antigens containing the greater concentrations of HCl. Acid agglutination was apparently accelerated by the presence of bovine serum. That is, the antigens containing the larger concentrations of acid did not agglutinate by themselves but did agglutinate when bovine sera that was negative to *Bact. abortus* antigens was added to the antigen. A similar relation of serum to acid agglutination is reported by Karstner and Ecker (12).

The titres of agglutinating sera were very slightly inhibited when the antigens with which they were tested contained sufficient NaOH to result in pH values between 9.1 and 9.6. However, the agglutination in such antigens was frequently atypical and stringy in appearance instead of the usual clumps of serum agglutinated bacteria.

Summary

These results indicate that the titres of agglutinating sera are not appreciably influenced by a broad zone in pH values of *Bact. abortus* antigens (concentration 0.04% bacteria by centrifuge tube method), such variations in pH resulting from the addition of dilute solutions of HCl and NaOH to the antigen. The limits of pH values of antigens in which the titres of agglutinating sera remained unaltered were approximately 4.7 to 8.9.

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XIII. EFFECT OF VARIATIONS IN CONCENTRATION OF SODIUM CHLORIDE IN *BACT. ABORTUS* ANTIGENS ON THE AGGLUTINATION REACTIONS OF BOVINE SERA

Many laboratories employ 0.85% NaCl solution in the preparation of *Bact. abortus* antigen for use in the test tube agglutination test for the diagnosis of Bang's disease of animals or undulant fever in man. This concentration has been satisfactory. These studies were conducted to determine the influence of other concentrations of NaCl in the antigen on the serum-agglutination reaction.

It has long been known that salt (electrolyte) in agglutination antigens is essential for serum agglutination. Buchanan and Fulmer (1) discuss, in part, the characteristics and action of agglutinins as follows: "The sensitizing substance developed in an animal body as the result of the introduction of a suspension of cells is termed a specific agglutinin. Apparently the action of the agglutinin is specifically to sensitize the homologous antigen (cell) to the action of electrolytes. Bordet (1896) clearly recognized and proved that in specific agglutination there are two distinct phases, first, the impression or sensitization of the bacterial cell by the agglutinins and second the precipitation or agglutination of the cells due to the action of salts." In regard to salt agglutination of cells they state in part as follows: "Apparently two distinct effects of salts upon micro-organisms are to be differentiated. One is the so-called *salting out* effect produced by relatively high concentrations of certain salts, the other is the zonal agglutination of the same or other salts at much lower concentrations. The concentration of salt

required to salt out bacteria from a suspension varies with the salt used, the presence or absence of other constituents, and the character of the organism. The phenomenon of so-called spontaneous agglutination of bacteria is worthy of note. It occasionally happens that a suspension of an organism usually not agglutinable by moderate salt concentrations becomes very readily agglutinable so that it is sensitive to the concentration of sodium chloride present in physiological salt solution. In general, with bacteria, univalent ions are much less potent in causing agglutination than are divalent or polyvalent ions."

Huddleson and Abell (2) recommend a 12% solution of NaCl for use in the preparation of concentrated *Bact. abortus* antigen for the rapid macroscopic test for the diagnosis of Bang's disease in animals or undulant fever in man.

In our experiments bacteria were grown on nutrient agar slants which contained 0.5% NaCl. The organisms were washed from the agar slants with sterile distilled water to which 0.5% phenol had been added as a preservative. The concentration of bacteria in the suspension was adjusted to 0.04% by the centrifuge tube method (loc. cit.). Subsequently the suspension of organisms was divided in several flasks and various concentrations of NaCl were added to the antigens. Agglutination tests with bovine sera having a wide range of agglutinin content were conducted with the antigens having different concentrations of NaCl. The technic of preparing the serum-antigen dilutions was the same as previously. All agglutination tests were held in the incubator at 37.5° C., and observations made at 24, 48, and 72 hours to record the rate of agglutination and the maximum titres of the agglutinating sera. The maximum titres of all of the sera were determined with all antigens. The polyvalent stock antigen containing 0.85% NaCl was used as a check on the maximum titres of the agglutinating sera.

Each of five series of antigens with a range of concentration of NaCl were tested with a group of bovine sera having a wide range of agglutinin content. Table 1 shows the number of antigens in each series, the concentration of NaCl in each antigen, and the number and agglutinin content of the bovine sera with which they were tested.

Table 1

Number and Concentration of NaCl in Antigens and the Number and Agglutinin Content of Sera Tested

Series No.	No. of antigens in series	Per cent concentration of NaCl in antigens	No. of bovine sera tested	Sera with high agglutinin content, titre 1:500 or above	Sera with low to medium agglutinin content, titre 1:25 to 1:100	Sera with no agglutinin content
1	7	0.85, 2, 5, 10, 12, 15, 20	8	2	5	1
2	15	0.85, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20	6	4	1	1
3	8	0.0, 0.05, 0.1, 0.2, 0.25, 0.3, 0.5, 0.85	6	3	2	1
4	12	0.0, 0.05, 0.1, 0.25, 0.3, 0.5, 0.6, 0.75, 0.85, 0.9, 1.0, 1.25	7	3	3	1
5	12	Same as Series No. 4	11	6	4	1

Table 2 shows typical agglutination tests with representative series of antigens and agglutinating sera representing a wide range of agglutinin content.

Table 2

Typical Results of Agglutination Tests of Bovine Sera with Bact. Abortus Antigens Having Different Concentrations of NaCl

Per cent concentration of NaCl in antigen	Approximate time of observation	Dilutions: 1-25, 1-50, 1-100, 1-250, 1-500, 1-1000			
		Serum 130	Serum 533	Serum 128	Serum 21
0.0	24 hours	— — — — — —	— — — — — —	— — — — — —	— — — — — —
	48 "	— — — — — —	— — — — — —	— — — — — —	— — — — — —
	72 "	— — — — — —	I — — — — —	— — — — — —	— — — — — —
0.05	24 hours	— — — — — —	+ I I I — —	— — — — — —	— — — — — —
	48 "	I — — — — —	+ + I I — —	— — — — — —	— — — — — —
	72 "	+ — — — — —	+ + + + — —	— — — — — —	— — — — — —
0.10	24 hours	I — — — — —	+ + I I — —	— — — — — —	— — — — — —
	48 "	+ — — — — —	+ + + + I —	I — — — — —	— — — — — —
	72 "	+ I — — — —	+ + + + + —	+ — — — — —	— — — — — —
0.20	24 hours	+ — — — — —	+ + I I — —	I — — — — —	— — — — — —
	48 "	+ + — — — —	+ + + + + I	+ I — — — —	— — — — — —
	72 "	+ + — — — —	+ + + + + +	+ I — — — —	— — — — — —
0.25	24 hours	+ I — — — —	+ + + + + —	I I — — — —	— — — — — —
	48 "	+ + I — — —	+ + + + + —	+ I — — — —	— — — — — —
	72 "	+ + + — — —	+ + + + + —	+ I — — — —	— — — — — —
0.30	24 hours	+ I — — — —	+ + + + + —	I — — — — —	— — — — — —
	48 "	+ + I — — —	+ + + + + —	+ I — — — —	— — — — — —
	72 "	+ + + — — —	+ + + + + I	+ I — — — —	— — — — — —

\+ = Complete serum agglutination. c = Cloudy.
I = Incomplete serum agglutination. S = Stringy condition of agglutinated bacteria.
— = No agglutination. X = Salt agglutination.

Table 2—Continued

Typical Results of Agglutination Tests of Bovine Sera with Bact. Abortus Antigens Having Different Concentrations of NaCl

Per cent concentration of NaCl in antigen	Approximate time of observation	Dilutions: 1-25, 1-50, 1-100, 1-250, 1-500, 1-1000			
		Serum 130	Serum 533	Serum 128	Serum 21
0.50	24 hours	+ I – – – –	+ + + + I –	I – – – – –	– – – – – –
	48 "	+ + – – – –	+ + + + + –	+ I – – – –	– – – – – –
	72 "	+ + I – – –	+ + + + + –	+ I – – – –	– – – – – –
0.85	24 hours	+ + – – – –	+ + + + + –	+ – – – – –	– – – – – –
	48 "	+ + + – – –	+ + + + + –	+ I – – – –	– – – – – –
	72 "	+ + + – – –	+ + + + + I	+ I – – – –	– – – – – –
0.90	24 hours	+ + I – – –	+ + + + + –	+ – – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
	72 "	+ + + – – –	+ + + + + I	+ I I – – –	– – – – – –
1.0	24 hours	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
	72 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
1.25	24 hours	+ + I – – –	+ + + + I I	+ – – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
	72 "	+ + + – – –	+ + + + + I	+ I – – – –	– – – – – –
1.5	24 hours	+ + – – – –	+ + + + I I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
	72 "	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
2.0	24 hours	+ + – – – –	+ + + + I I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
	72 "	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
3.0	24 hours	+ + – – – –	+ + + + I I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
	72 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
4.0	24 hours	+ I – – – –	+ + + + I I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
	72 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
5.0	24 hours	+ I I – – –	+ + + + I I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + + I	+ + I – – –	– – – – – –
	72 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
6.0	24 hours	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + I I	+ + – – – –	– – – – – –
	72 "	+ + + – – –	+ + + + I I	+ I – – – –	– – – – – –
7.0	24 hours	+ + I – – –	+ + + + I I	+ – – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + I I	+ + I – – –	– – – – – –
	72 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
8.0	24 hours	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
	48 "	+ + I – – –	+ + + + + I	+ + – – – –	– – – – – –
	72 "	+ + I – – –	+ + + + + I	+ I – – – –	– – – – – –
			S S S S S S		
9.0	24 hours	+ + I – – –	+ + + + I I	+ I – – – –	– – – – – –
			S S S S S		
	48 "	+ + I – – –	I I + + I –	+ I – – – –	– – – – – –
		c c c	S S S S S		
	72 "	+ + I – – –	I I + + I –	+ I – – – –	– – – – – –

+ = Complete serum agglutination. c = Cloudy.
I = Incomplete serum agglutination. S = Stringy condition of agglutinated bacteria.
– = No agglutination. X = Salt agglutination.

Table 2—Continued
Typical Results of Agglutination Tests of Bovine Sera with Bact. Abortus Antigens Having Different Concentrations of NaCl

Per cent concentration of NaCl in antigen	Approximate time of observation	Dilutions: 1-25, 1-50, 1-100, 1-250, 1-500, 1-1000															
		Serum 130						Serum 533						Serum 128						Serum 21					
10.0	24 hours	I	I	I	—	—	—	S +	S +	S +	S +	S I	S I	+	—	—	—	—	—	—	—	—	—	—	—
								S	S	S	S	S	S												
	48 "	I	I	I	—	—	—	I	+	+	+	I	I	I	I	—	—	—	—	—	—	—	—	—	—
		c	c	c				S	S	S	S	S	S	c	c										
	72 "	+	+	I	—	—	—	I	+	+	+	I	I	+	I	—	—	—	—	—	—	—	—	—	—
								S	S	S	S	S													
11.0	24 hours	I	I	I	—	—	—	I	I	+	+	I	—	I	I	—	—	—	—	—	—	—	—	—	—
		c	c	c				S	S	S	S	S													
	48 "	I	I	I	—	—	—	I	+	+	+	I	—	I	I	—	—	—	—	—	—	—	—	—	—
		c	c	c				S	S	S	S	S		c	c										
	72 "	I	I	I	—	—	—	+	+	+	+	I	—	I	I	—	—	—	—	—	—	—	—	—	—
								S	S	S	S														
12.0	24 hours	+	I	I	—	—	—	I	I	+	+	—	—	I	—	—	—	—	—	—	—	—	—	—	—
		c	c	c				S	S	S	S	S													
	48 "	+	I	I	—	—	—	I	+	+	+	I	—	I	I	—	—	—	—	—	—	—	—	—	—
		c	c	c				S	S	S	S	S													
	72 "	I	I	I	—	—	—	I	+	+	+	I	—	I	I	—	—	—	—	—	—	—	—	—	—
								S	S	S	S	S													
15.0	24 hours	—	—	—	—	—	—	I	I	I	I	I	—	—	—	—	—	—	—	—	—	—	—	—	—
								S	S	S	S	S		X											
	48 "	I	I	I	—	—	—	I	+	+	+	I	—	+	—	—	—	—	—	—	—	—	—	—	—
		c	c	c				S	c	c	c	c		X	X	X									
	72 "	I	I	I	—	—	—	I	+	+	+	I	—	+	+	+	—	—	—	—	—	—	—	—	—
		X	X	X				S	S	S															
20.0	24 hours	+	+	+	—	—	—	I	I	I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		X	X	X				S	S	S				X	X	X	X	X	X	X	X	X	X	X	X
	48 "	I	I	I	—	—	—	I	I	I	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
		X	X	X				c	c	c				X	X	X	X	X	X	X	X	X	X	X	X
	72 "	+	+	+	—	—	—	I	I	I	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+

Per cent concentration of NaCl in antigen	Approximate time of observation	SERUM 153—Dilutions															
		1-25	1-50	1-100	1-250	1-500	1-1000	1-2000	1-3000	1-4000	1-5000	1-6000	1-7000	1-8000	1-9000	1-10000
0.0	24 hours	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	48 "	+	I	I	—	—	—	—	—	—	—	—	—	—	—	—
	72 "	+	I	I	—	—	—	—	—	—	—	—	—	—	—	—
0.05	24 hours	+	+	I	I	—	—	—	—	—	—	—	—	—	—	—
	48 "	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—
	72 "	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—
0.10	24 hours	+	+	+	I	—	—	—	—	—	—	—	—	—	—	—
	48 "	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
	72 "	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
0.25	24 hours	+	+	+	+	+	+	+	+	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—

+ = Complete serum agglutination.
I = Incomplete serium agglutination.
— = No agglutination.
c = Cloudy.
S = Stringy condition of agglutinated bacteria.
X = Salt agglutination.

Table 2—Continued

Typical Results of Agglutination Tests of Bovine Sera with Bact. Abortus Antigens Having Different Concentrations of NaCl

Per cent concentration of NaCl in antigen	Approximate time of observation	SERUM 153—Dilutions													
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000	1–6000	1–7000	1–8000	1–9000	1–10000
0.30	24 hours	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
0.50	24 hours	+	+	+	+	+	+	+	I	+	—	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
0.60	24 hours	+	+	+	+	+	+	+	+	+	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
0.75	24 hours	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
0.85	24 hours	+	+	+	+	+	+	+	I	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
0.90	24 hours	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
1.0	24 hours	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
1.25	24 hours	+	+	+	+	+	+	+	I	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
1.50	24 hours	+	+	+	+	+	+	+	I	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
2.0	24 hours	+	+	+	+	+	+	+	I	I	I	I	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	I	I	—	—	—
3.0	24 hours	+	+	+	+	+	+	+	+	I	I	I	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
4.0	24 hours	+	+	+	+	+	+	+	+	I	I	I	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
5.0	24 hours	+	+	+	+	+	+	+	+	I	I	—	I	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	—	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	I	+	I	—	—
6.0	24 hours	+	+	+	+	+	+	+	+	I	I	I	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
7.0	24 hours	+	+	+	+	+	+	+	+	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—

+ = Complete serum agglutination. c = Cloudy.
I = Incomplete serum agglutination. S = Stringy condition of agglutinated bacteria.
— = No agglutination. X = Salt agglutination.

Table 2—Continued
Typical Results of Agglutination Tests of Bovine Sera with Bact. Abortus Antigens Having Different Concentrations of NaCl

Per cent antigen tration of NaCl in antigen	Approx-tion time of observa-tion	SERUM 153—Dilutions														
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000	1–6000	1–7000	1–8000	1–9000	1–10000
8.0	24 hours	+	+	+	+	+	+	+	I	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
		S	S	S	S	S	S	S	S	S	S					
9.0	24 hours	+	+	+	+	+	+	+	+	I	I	—	—	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S			
	48 "	+	+	+	+	+	+	+	+	+	I	+	+	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S	S		
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	I	—	—
		S	S	S	S	S	S	S	S	S	S					
10.0	24 hours	+	+	+	+	+	+	+	I	I	I	—	—	—	—	—
		S	S	S	S	S	S	S	S	S	S		S			
	48 "	+	+	+	+	+	+	+	+	+	+	—	I	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S	S		
	72 "	+	+	+	+	+	+	+	+	+	+	I	+	I	—	—
		S	S	S	S	S	S	S	S	S	S					
11.0	24 hours	+	+	+	+	+	+	+	+	+	I	—	—	—	—	—
		S	S	S	S	S	S	S	S	S	S		S			
	48 "	+	+	+	+	+	+	+	+	+	+	—	I	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S			
	72 "	+	+	+	+	+	+	+	+	+	+	I	+	—	—	—
		S	S	S	S	S	S	S	S	S						
12.0	24 hours	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S			
	48 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S			
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
		S	S	S	S	S	S	S	S							
15.0	24 hours	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S			
	48 "	+	+	+	+	+	+	+	+	+	I	I	I	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S			
	72 "	+	+	+	+	+	+	+	+	+	I	I	I	—	—	—
		S	S	S	S	S	S	S	S							
20.0	24 hours	+	+	+	+	I	I	I	I	—	—	—	—	—	—	—
		S	S	S	S	S	S	S	S	S	S					
	48 "	+	+	+	+	+	+	I	I	I	I	—	—	—	—	—
		S	S	S	S	S	S	S	S	S	S	S	S			
	72 "	+	+	+	+	+	+	+	+	+	I	I	I	—	—	—

+ = Complete serum agglutination. c = Cloudy.
I = Incomplete serum agglutination. S = Stringy condition of agglutinated bacteria.
— = No agglutination. X = Salt agglutination.

Results

The agglutination reactions of bovine sera tested with antigen having a concentration of NaCl between approximately 0.25% and 8% were not appreciably altered either with regard to rate of agglutination, maximum titres of the agglutinating sera, or character of the

clumps of agglutinated bacteria. When the antigen contained a concentration of NaCl less than 0.25% there was usually a marked inhibition of the agglutination reaction both in rate of agglutination and maximum titres of the agglutinating sera. There was usually no visible agglutination in antigens to which no salt had been added. In a few instances there was some agglutination in the lowest dilutions when such antigens were tested with high agglutinin content sera. In these cases it is assumed that the antigen did have a very weak concentration of salt, possibly dissolved from the agar slants in the process of washing the bacteria from them with distilled water, or salt from the blood serum.

The reactions of agglutinating sera were inhibited when tested with antigens having a concentration of NaCl of 9% or more. In such antigens the maximum titres were somewhat lower than the titres of the same agglutinating sera with standard stock antigen, and the clumps of agglutinated bacteria were stringy in appearance.

A salting out of the bacteria was observed in serum-antigen mixtures having a concentration of 15% or more of NaCl. This phenomenon did not occur in the antigens until after serum was added but was present in mixtures of such antigens with bovine serum having no specific agglutinin for *Bact. abortus* antigen.

Summary

The reactions of agglutinating sera used in the test tube agglutination test were not appreciably altered when tested with *Bact. abortus* antigens (0.04% bacteria by centrifuge tube method) having concentrations of NaCl between about 0.25% and 8%. An inhibition of the agglutination reaction was observed when the antigens had less than 0.25% or more than 8% concentration of NaCl.

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XIV. INFLUENCE OF TEMPERATURE ON THE RATE OF AGGLUTINATION OF *BACT. ABORTUS* ANTIGENS IN THE TEST TUBE AGGLUTINATION TEST FOR BANG'S DISEASE

No uniform procedure of incubating agglutination tests for the diagnosis of Bang's disease in animals or undulant fever in man has been adopted by the various laboratories conducting such tests.

Some laboratories hold the tests at room temperature; others place them in hot-air incubators or water-baths at temperatures ranging up to 55° C. Still other laboratories incubate the tests for short periods of time and then place them either in a refrigerator or at room temperature for an additional period before final observation of the tests. Likewise, there is no uniformity in the time interval before the final observation of the tests. Some workers make the final reading on the morning of the day following which the tests were prepared. In other laboratories the final reading occurs on the second day and in still other laboratories the final observation of the tests is made on the third day.

Fleming (1) states that the following sums up the matter of relation of temperature to rate of agglutination: "It occurs more rapidly as the temperature rises up to an optimum of 55° C. The agglutination reaction as we do it comprises two quite different processes—firstly the union of the agglutinin of the serum with the bacteria, and secondly the clumping of the bacteria. The work of Gaehtgens (1906, 1908) and Gates (1922) has shown that the first process, the union of agglutinin and the bacterium, takes place very rapidly, and that by far the greater portion of the time in the ordinary reaction is occupied by the sensitized bacteria coming together in clumps."

Henry and Traum (2) compared six different procedures of incubation of agglutination tests with *Bact. abortus* antigen as follows:

1. Overnight at 37.5° C., 24 hours at room temperature.
2. Two hours at 37.5° C., icebox overnight.
3. Four hours at 37.5° C., icebox overnight.
4. Four hours at 55° C., icebox overnight.
5. Seventy-two hours at room temperature.
6. One-half hour at 37.5° C., centrifugated at 1,600 r.p.m. for ten minutes.

They state: "Incubation overnight at 37.5° C., and then for twenty-four hours at room temperature gives better results than other methods of holding described in this paper. The tests, altho performed on only a small number of sera, indicate that while procedures 2, 3, and 4 have the apparent advantage of saving a day in obtaining results, none yielded as good results as did procedure 1. Even the time-saving ele-

ment is not present, since the first readings with procedure 1 (after incubation at 37.5° C., overnight) were better than, or as good as, the final readings with procedures 2, 3, and 4. Procedure 5 gave results nearly equal to those of No. 1, but had the disadvantage of requiring one day longer for the completion of the test. Procedure 6 yielded as high titres for the sera as any. The physical equipment for the performance of several hundred tests in a day makes this method unsuitable for many laboratories."

In a previous publication (3) the results were reported of a study of the rate of agglutination at 37.5° C. in approximately 8,000 agglutination tests. This work indicated that there are differences in the rapidity of agglutination of different sera when tested with the same antigen. That is, some sera attain the maximum agglutination titre much more rapidly than do others having a similar maximum titre with the same antigen and incubation conditions. Further, that such differences in the rapidity of agglutination are not confined to any group of sera with regard to agglutinin content. That is, such differences in the rate of agglutination were observed with sera having a wide range of agglutinin content and consequent wide variations in maximum agglutination titres.

It was observed that the maximum titres of sera of high agglutinin content were not always attained until the fourth day at 37.5° C. However, sera of high agglutinin content (titre 1:250 or above) usually showed agglutination, in the dilutions employed in routine diagnosis work, after 24 hours at 37.5° C. Consequently the final observation of tests of sera of high agglutinin content in routine diagnosis dilutions could usually be made after 24 hours. It has been shown (3) that approximately 22% of the sera received at this laboratory for the diagnosis of Bang's disease contained sufficient agglutinins to be classified as positive and are therefore considered in the group of sera of high agglutinin content, the final observations of tests of which are usually satisfactory after 24 hours at 37.5° C. This work also showed that the maximum titres of sera of low to medium agglutinin content (titre 1:25 to 1:100) were not always attained after 24 hours at 37.5° C., and consequently later observations were necessary. The sera of low to medium agglutinin content was shown to represent approximately 5% of the total number of samples received in this laboratory for routine diagnosis; which, in turn, is approximately 20% of the total number of serum samples in which agglutinins were demonstrated. It was further observed that while not all the samples of low to medium agglutinin content attained their maximum titres after 24 hours at 37.5° C., some did and the 24-hour observation was therefore in agreement with later observations. It appears, therefore, from a practical

point of view, that the importance of the interval of time before final observation of tests incubated at 37.5° C. is confined to only a part of approximately 5% of all serum samples received in this laboratory for diagnosis. This is a part of approximately 20% of the serum samples in which agglutinins can be demonstrated. These findings, it is believed, are substantiated by the experiences of other laboratories conducting routine tests for diagnosis, using different systems of incubation and different intervals before final observation of agglutination tests without any major discrepancies in their results that are attributable to these differences in technic of conducting the tests.

With these findings in mind, experiments were conducted in which several different systems of incubation of agglutination tests were utilized, not alone to compare the rate of agglutination in the different incubation procedures but also to study the relation between a given procedure of incubation and the desirable time of final observation of agglutination tests. In this latter regard, only the dilutions usually employed in routine diagnosis were considered. The importance of this phase of the problem is necessarily dependent on some classification of bovine sera relative to their agglutinin content and consequent maximum titre. These are for this work as follows: sera of high agglutinin content (titre 1:250 or above), of low to medium agglutinin content (titre 1:25 to 1:100), and no agglutinin content.

In one series of experiments the following procedures of incubation of agglutination tests were compared.

1. Hot-air incubator 37.5° C.
2. Room temperature.
3. Refrigerator 3.5° C.
4. Water-bath at 37.5° C 4 hours—refrigerator 3.5° C.
5. Water-bath at 37.5° C., overnight—incubator 37.5° C.
6. Water-bath at 37.5° C., overnight—room temperature.
7. Water-bath at 37.5° C., overnight—refrigerator 3.5° C.
8. Water-bath 37.5° C., continuously for 72 hours.

The agglutination tests were observed at approximately 24, 48, and 72 hours, and the results recorded. Thirty-six bovine sera were used containing *Bact. abortus* agglutinins and representing a wide range in agglutinin content. Sufficient dilutions were prepared to determine the maximum titres of all of the sera. The stock polyvalent antigen (0.04% bacteria by centrifuge tube method, 0.5% phenol as preservative) was used for all tests. The technic of preparing the serum antigen dilutions was the same as that previously described (3). Table 1 gives typical results of tests of 5 sera in this series of experiments.

Table 1
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 144—Dilutions									
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000
Incubator at 37.5° C.	24 hours	+	+	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
Room temperature	24 hours	+	+	+	+	+	I	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
Water-bath at 37.5° C.	24 hours	+	+	+	+	+	—	—	—	—	—
	48 "	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
Refrigerator at 3.5° C.	24 hours	+	+	+	+	I	I	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
Water-bath at 37.5° C. Overnight—room temperature	24 hours	+	+	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
Water-bath at 37.5° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
Water-bath at 37.5° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
Water-bath at 37.5° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	I	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—
		SERUM 142									
Incubator at 37.5° C.	24 hours	+	+	+	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	I	I	—	—
Room temperature	24 hours	+	+	I	+	I	—	—	—	—	—
	48 "	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	I	—	—	—
Water-bath at 37.5° C.	24 hours	+	+	+	+	I	—	—	—	—	—
	48 "	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	I	I	—	—
Refrigerator at 3.5° C.	24 hours	+	+	+	+	+	I	—	—	—	—
	48 "	+	+	+	+	+	+	I	—	—	—
	72 "	+	+	+	+	+	+	+	—	—	—
Water-bath at 37.5° C. Overnight—room temperature	24 hours	+	+	+	+	—	—	—	—	—	—
	48 "	+	+	+	+	I	—	—	—	—	—
	72 "	+	+	+	+	+	I	—	—	—	—
Water-bath at 37.5° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	I	—	—	—	—	—
	48 "	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	I	I	—	—	—
Water-bath at 37.5° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—	—	—	—	—
	48 "	+	+	+	+	I	I	—	—	—	—
	72 "	+	+	+	+	+	I	I	—	—	—
Water-bath at 37.5° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	I	—	—	—	—	—	—
	48 "	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	I	—	—	—	—

Table 1—Continued
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 8—Dilutions					
		1–25	1–50	1–100	1–250	1–500	1–1000
Incubator at 37.5° C.	24 hours	+	+	+	I	—	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	I	—
Room temperature	24 hours	+	+	I	—	—	—
	48 "	+	+	+	I	—	—
	72 "	+	+	+	+	—	—
Water-bath at 37.5° C.	24 hours	+	+	+	I	—	—
	48 "	+	+	+	+	—	—
	72 "	+	+	+	+	I	—
Refrigerator at 3.5° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	+	I	—	—
	72 "	+	+	+	I	—	—
Water-bath at 37.5° C. Overnight—room temperature	24 hours	+	+	+	I	—	—
	48 "	+	+	+	+	—	—
	72 "	+	+	+	+	I	—
Water-bath at 37.5° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	I	—
Water-bath at 37.5° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	—	—
	72 "	+	+	+	+	—	—
Water-bath at 37.5° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	—	—
	72 "	+	+	+	+	I	—
		SERUM 128					
Incubator at 37.5° C.	24 hours	+	—	—	—	—	—
	48 "	+	I	—	—	—	—
	72 "	+	I	—	—	—	—
Room temperature	24 hours	+	I	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 37.5° C.	24 hours	+	I	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Refrigerator at 3.5° C.	24 hours	I	I	—	—	—	—
	48 "	+	I	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 37.5° C. Overnight—room temperature	24 hours	+	I	—	—	—	—
	48 "	+	I	—	—	—	—
	72 "	+	I	—	—	—	—
Water-bath at 37.5° C. Overnight—incubator at 37.5° C.	24 hours	+	I	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 37.5° C. Overnight—refrigerator at 3.5° C.	24 hours	+	I	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 37.5° C. Four hours—refrigerator at 3.5° C.	24 hours	+	I	—	—	—	—
	48 "	+	I	—	—	—	—
	72 "	+	+	—	—	—	—

Table 1—Continued
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 26—Dilutions					
		1–25	1–50	1–100	1–250	1–500	1–1000
Incubator at 37.5° C.	24 hours	+	+	I	—	—	—
	48 "	+	+	+	I	—	—
	72 "	+	+	+	I	—	—
Room temperature	24 hours	+	+	+	—	—	—
	48 "	+	+	+	—	—	—
	72 "	+	+	+	I	—	—
Water-bath at 37.5° C.	24 hours	+	+	+	—	—	—
	48 "	+	+	+	—	—	—
	48 "	+	+	+	—	—	—
Refrigerator at 3.5° C.	24 hours	+	I	—	—	—	—
	48 "	+	+	I	—	—	—
	72 "	+	+	I	I	—	—
Water-bath at 37.5° C. Overnight—room temperature	24 hours	+	+	+	—	—	—
	48 "	+	+	+	—	—	—
	72 "	+	+	+	—	—	—
Water-bath at 37.5° C. Overnight—incubator at 37.5° C.	24 hours	+	+	I	—	—	—
	48 "	+	+	I	—	—	—
	72 "	+	+	+	—	—	—
Water-bath at 37.5° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	—	—	—
	48 "	+	+	+	—	—	—
	72 "	+	+	+	—	—	—
Water-bath at 37.5° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	—	—	—
	48 "	+	+	+	—	—	—
	72 "	+	+	+	—	—	—

Results

There was no appreciable difference between the results of tests held in a hot-air incubator at 37.5° C. and the duplicate tests held in a water-bath at 37.5° C. When the tests had been held in a water-bath at 37.5° C. overnight, removal to room temperature, hot-air incubator at 37.5° C., or refrigerator at 3.5° C. apparently did not materially influence the subsequent rate or extent of agglutination.

In general, the rate of agglutination of tests at room temperatures was appreciably slower than duplicate tests held at 37.5° C. However, the maximum titres of sera with a wide range of agglutinin content were essentially the same after 72 hours in the tests at room temperature and at 37.5° C. in either a hot-air incubator or a water-bath.

The rate of agglutination was definitely slower in tests held continuously in the refrigerator at 3.5° C. The maximum titres, particularly with sera of high agglutinin content, were definitely lower after 72 hours than duplicate tests that had been incubated either at 37.5° C. or held at room temperature.

The findings, previously mentioned, relative to differences in rapidity of agglutination of different sera at 37.5° C. were again encountered

and found in all these various procedures of incubation of agglutination tests.

In the second series of experiments, the same eight procedures of incubating agglutination tests were carried out, except that the water-bath was held at approximately 55° C. Thirty-four bovine sera containing *Bact. abortus* agglutinins with a wide range of agglutinin content were used. Table 2 gives typical results of tests of five sera in this series of experiments.

Table 2
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 2—Dilutions					
		1–25	1–50	1–100	1–250	1–500	1–1000
Incubator at 37.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	I
Room temperature	24 hours	+	+	+	+	—	—
	48 "	+	+	+	I	I	—
	72 "	+	+	+	+	+	—
Water-bath at 55° C.	24 hours	+	+	+	+	+	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	—
Refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	I
Water-bath at 55° C. Overnight—room temperature	24 hours	+	+	+	+	+	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	—
Water-bath at 55° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	+	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	+
Water-bath at 55° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	—
Water-bath at 55° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	I	I
	48 "	+	+	+	+	+	I
	72 "	+	+	+	+	+	I
		SERUM 533					
Incubator at 37.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	+	—
Room temperature	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	I	—
Water-bath at 55° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	I
Refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	—
Water-bath at 55° C. Overnight—room temperature	24 hours	+	+	+	+	+	—
	48 "	+	+	+	+	+	I
	72 "	+	+	+	+	+	I

Table 2—Continued
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 533—Dilutions 1–25	1–50	1–100	1–250	1–500	1–1000
Water-bath at 55° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	+	—
Water-bath at 55° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	I	—
Water-bath at 55° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—
	48 "	+	+	+	+	—	—
	72 "	+	+	+	+	+	I
		SERUM 130					
Incubator at 37.5° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	I	—	—	—
Room temperature	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 55° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Refrigerator at 3.5° C.	24 hours	+	+	I	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	+	—	—	—
Water-bath at 55° C. Overnight—room temperature	24 hours	+	+	I	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 55° C. Overnight—incubator at 37.5° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 55° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 55° C. Four hours—refrigerator at 3.5° C.	24 hours	+	I	I	—	—	—
	48 "	+	+	I	—	—	—
	72 "	+	+	I	—	—	—

Procedure of incubation of tests	Approximate time of observation	SERUM 410—Dilutions 1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000	1–6000
Incubator at 37.5° C.	24 hours	+	+	+	+	+	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	+	I	—	—	—
Room temperature	24 hours	+	+	+	+	+	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	+	I	—	—	—
Water-bath at 55° C.	24 hours	+	+	+	+	+	+	+	+	I	—	—
	48 "	+	+	+	+	+	+	+	+	—	—	—
	72 "	+	+	+	+	+	+	+	+	—	—	—
Refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	—	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—	—
	72 "	+	+	+	+	+	+	—	—	—	—	—

Table 2—Continued
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 410—Dilutions										
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000	1–6000
Water-bath at 55° C. Overnight—room temperature	24 hours	+	+	+	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	I	—	I	—	—
	72 "	+	+	+	+	+	+	I	+	+	—	—
Water-bath at 55° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	+	+	+	+	I	—	—
	48 "	+	+	+	+	+	+	+	I	I	—	—
	72 "	+	+	+	+	+	+	+	+	+	—	—
Water-bath at 55° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	+	+	I	—	—
	48 "	+	+	+	+	+	+	+	+	+	—	—
	72 "	+	+	+	+	+	+	+	+	+	—	—
Water-bath at 55° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	+	+	+	—	—
	72 "	+	+	+	+	+	+	+	+	+	—	—

Procedure of incubation of tests	Approximate time of observation	SERUM 137—Dilutions														
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000	1–6000	1–7000	1–8000	1–9000	1–10000	1–11000
Incubator at 37.5° C.	24 hours	+	+	+	+	+	+	+	+	I	I	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
Room temperature	24 hours	+	+	+	+	I	—	—	—	—	—	—	—	—	—	—
	48 "	+	+	+	+	I	I	I	I	I	—	—	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	I	I	I	—	—	—
Water-bath at 55° C.	24 hours	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	I	—	—
Refrigerator at 3.5° C.	24 hours	+	+	+	+	+	I	+	—	—	—	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
	72 "	+	+	+	+	+	+	+	I	—	—	—	—	—	—	—
Water-bath at 55° C. Overnight—room temperature	24 hours	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	I	I	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
Water-bath at 55° C. Overnight—incubator 37.5° C.	24 hours	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	I	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	I	+	+	—
Water-bath at 55° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	+	+	+	+	+	+	I	—	I
	48 "	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—
Water-bath at 55° C. Overnight—4 hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	+	+	—	+	—	—
	72 "	+	+	+	+	+	+	+	+	+	+	+	—	+	—	—

Results

The results of these experiments were much the same as those reported in the previous series of experiments, in which the water-bath temperature was 37.5°C. However, the following differences were observed. The rate of agglutination was usually faster at 55° than at 37.5°C. In this series of experiments, some samples of sera in each of the various ranges of agglutinin content did not attain their maximum agglutination titres after 24 hours at 55°C. The temperature of the water-bath fluctuated somewhat, owing to the thermo-regulating mechanism and at times reached 58°C. Such temperatures were attended by some inhibition of the agglutination reaction of some of the sera.

The third series of experiments employed the same procedures of incubation of agglutination tests as reported before, except that the water-bath was adjusted to 50°C. Forty-two bovine sera containing *Bact. abortus* agglutinins with a wide range of agglutinin content were used. Table 3 gives typical results of tests of five sera in this series of experiments.

Table 3

Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 153—Dilutions									
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000
Incubator at 37.5° C.	24 hours	+	+	+	+	+	+	I	—	—	—
	48 "	+	+	+	+	+	+	+	—	—	—
	72 "	+	+	+	+	+	+	+	I	—	—
Room temperature	24 hours	+	+	+	+	I	I	—	—	—	—
	48 "	+	+	+	+	+	+	I	—	—	—
	72 "	+	+	+	+	+	+	+	—	—	—
Water-bath at 50° C.	24 hours	+	+	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	+	+	I	—
	72 "	+	+	+	+	+	+	+	+	I	—
Refrigerator at 3.5° C.	24 hours	+	+	+	+	I	—	—	—	—	—
	48 "	+	+	+	+	+	I	—	—	—	—
	72 "	+	+	+	+	+	+	I	—	—	—
Water-bath at 50° C. Overnight—room temperature	24 hours	+	+	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	+	+	I	—
	72 "	+	+	+	+	+	+	+	+	I	—
Water-bath at 50° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	+	—	—	—
	72 "	+	+	+	+	+	+	+	I	—	—
Water-bath at 50° C. night—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	I	—	—	—
	48 "	+	+	+	+	+	+	I	I	—	—
	72 "	+	+	+	+	+	+	+	I	—	—
Water-bath at 50° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	I	—	—	—
	48 "	+	+	+	+	+	+	+	I	—	—
	72 "	+	+	+	+	+	+	+	I	I	—

Table 3—Continued
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 142—Dilutions									
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000
Incubator at 37.5° C.	24 hours	+	+	+	+	+	—	—	—	—	—
	48 "	+	+	+	+	+	+	—	—	—	—
	72 "	+	+	+	+	+	+	+	+	+	I
Room temperature	24 hours	+	+	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	+	+	I	—	—
	72 "	+	+	+	+	+	+	+	+	—	—
Water-bath at 50° C.	24 hours	+	+	+	+	+	I	—	—	I	—
	48 "	+	+	+	+	+	+	I	I	+	+
	72 "	+	+	+	+	+	+	+	+	+	+
Refrigerator at 3.5° C.	24 hours	+	+	+	+	I	I	—	—	—	—
	48 "	+	+	+	+	+	+	+	—	—	—
	72 "	+	+	+	+	+	+	+	I	I	I
Water-bath at 50° C. Overnight—room temperature	24 hours	+	+	+	+	+	I	—	—	I	—
	48 "	+	+	+	+	+	+	I	I	+	+
	72 "	+	+	+	+	+	+	+	+	+	+
Water-bath at 50° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	+	—	—	—	+	—
	48 "	+	+	+	+	+	I	I	+	I	I
	72 "	+	+	+	+	+	+	+	+	+	+
Water-bath at 50° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	—	—	—	—	—
	48 "	+	+	+	+	+	+	+	+	+	I
	72 "	+	+	+	+	+	+	+	+	+	I
Water-bath at 50° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	I	—	—	—	—
	48 "	+	+	+	+	+	+	I	—	—	—
	72 "	+	+	+	+	+	+	+	—	—	—

Procedure of incubation of tests	Approximate time of observation	SERUM 185—Dilutions							
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000
Incubator at 37.5° C.	24 hours	+	+	+	+	+	I	—	—
	48 "	+	+	+	+	+	+	—	—
	72 "	+	+	+	+	+	+	—	—
Room temperature	24 hours	+	+	+	+	I	—	—	—
	48 "	+	+	+	+	+	I	—	—
	72 "	+	+	+	+	+	+	I	—
Water-bath at 50° C.	24 hours	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	—	—
	72 "	+	+	+	+	+	+	—	—
Refrigerator at 3.5° C.	24 hours	+	+	+	+	—	—	—	—
	48 "	+	+	+	+	+	—	—	—
	72 "	+	+	+	+	+	I	—	—
Water-bath at 50° C. Overnight—room temperature	24 hours	+	+	+	+	+	—	—	—
	48 "	+	+	+	+	+	—	—	—
	72 "	+	+	+	+	+	I	—	—
Water-bath at 50° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	+	I	—	—
	48 "	+	+	+	+	+	I	—	—
	72 "	+	+	+	+	+	I	—	—
Water-bath at 50° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	+	—	—
	48 "	+	+	+	+	+	+	—	—
	72 "	+	+	+	+	+	+	—	—
Water-bath at 50° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	+	—	—	—
	48 "	+	+	+	+	+	I	—	—
	72 "	+	+	+	+	+	I	—	—

Table 3—Continued
Results of Different Procedures of Incubation of Agglutination Tests

Procedure of incubation of tests	Approximate time of observation	SERUM 533—Dilutions					
		1–25	1–50	1–100	1–250	1–500	1–1000
Incubator at 37.5° C.	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	—
Room temperature	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	I
Water-bath at 50° C.	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	+	—
Refrigerator at 3.5° C.	24 hours	I	I	I	I	—	—
	48 "	+	+	+	+	+	—
	72 "	+	+	+	+	+	—
Water-bath at 50° C. Overnight—room temperature	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	I	—
Water-bath at 50° C. Overnight—incubator at 37.5° C.	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	+	—
Water-bath at 50° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	+	—
Water-bath at 50° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	+	+	I	—
	48 "	+	+	+	+	I	—
	72 "	+	+	+	+	+	—
		SERUM 130					
Incubator at 37.5° C.	24 hours	+	I	—	—	—	—
	72 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Room temperature	24 hours	+	+	—	—	—	—
	48 "	+	+	I	—	—	—
	72 "	+	+	+	—	—	—
Water-bath at 50° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Refrigerator at 3.5° C.	24 hours	—	—	—	—	—	—
	48 "	+	+	I	—	—	—
	72 "	+	+	I	—	—	—
Water-bath at 50° C. Overnight—room temperature	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 50° C. Overnight—incubator at 37.5° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 50° C. Overnight—refrigerator at 3.5° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—
Water-bath at 50° C. Four hours—refrigerator at 3.5° C.	24 hours	+	+	—	—	—	—
	48 "	+	+	—	—	—	—
	72 "	+	+	—	—	—	—

Results

The results were the same as those in the previous series of experiments, in which the temperature of the water-bath was 55°C., except that any fluctuations in the temperature of the water-bath which may have occurred were not sufficient to produce any unfavorable effects on the activity of the agglutinin. A recording thermometer was not used. These experiences suggest, however, that unless very accurate precision equipment is used when the tests are incubated at high temperatures, it is advisable to hold agglutination tests at 50°C. rather than at 55°C. The latter temperature is dangerously near a temperature at which agglutinin in some samples of bovine sera is altered in its activity. Any advantages of a water-bath at 55° C. as compared to one at 50°C. for incubating agglutination tests were not appreciable in these experiments.

The fourth series of experiments compared the rate and extent of agglutination with tests of duplicate samples of sera incubated in hot-air incubators at 37.5°, 40°, 46°, 48°, and 54°C. Thirty-six bovine sera were used containing *Bact. abortus* agglutinins and having a wide range of agglutinin content.

Table 4 gives typical results of tests of five sera in this series of experiments.

Table 4
Effect of Different Incubation Temperatures on the Rate and Extent of Agglutination of Bovine Sera

Temperature of incubator	Time of observation	SERUM 8—Dilutions									
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000
37.5° C.	17 hours	+	+	+	+	+	I	—	—	—	—
	43 "	+	+	+	+	+	+	I	—	—	—
	67 "	+	+	+	+	+	+	+	+	I	—
40° C.	17 hours	+	+	+	+	+	I	I	—	—	—
	43 "	+	+	+	+	+	+	+	—	—	—
	67 "	+	+	+	+	+	+	+	+	+	—
46° C.	17 hours	+	+	+	+	+	I	I	—	—	—
	43 "	+	+	+	+	+	+	+	I	—	—
	67 "	+	+	+	+	+	+	+	I	—	—
48° C.	17 hours	+	+	+	+	+	+	I	I	—	—
	43 "	+	+	+	+	+	+	+	+	—	—
	67 "	+	+	+	+	+	+	+	+	+	—
54° C.	17 hours	+	I	I	I	I	+	I	+	—	—
	43 "	+	+	+	+	+	+	+	+	—	—
	67 "	+	+	+	+	+	+	+	+	—	—

Table 4—Continued
Effect of Different Incubation Temperatures on the Rate and Extent of Agglutination of Bovine Sera

Temperature of incubator	Time of observation	SERUM 5—Dilutions									
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000
37.5° C.	17 hours	+	+	+	+	—	—	—	—	—	—
	43 "	+	+	+	+	+	I	—	—	—	—
	67 "	+	+	+	+	+	+	I	—	—	—
40° C.	17 hours	+	+	+	+	I	—	—	—	—	—
	43 "	+	+	+	+	I	—	—	—	—	—
	67 "	+	+	+	+	+	I	I	—	—	—
46° C.	17 hours	I	+	+	I	—	—	—	—	—	—
	43 "	+	+	+	+	I	I	—	—	—	—
	67 "	+	+	+	+	+	+	+	I	—	—
48° C.	17 hours	+	+	+	I	—	—	—	—	—	—
	43 "	+	+	+	+	—	—	—	—	—	—
	67 "	+	+	+	+	+	+	I	I	—	—
54° C.	17 hours	+	+	I	I	I	I	—	—	—	—
	43 "	+	+	+	+	I	I	I	—	—	—
	67 "	+	+	+	+	+	+	+	I	—	—

Temperature of incubator	Time of observation	SERUM 4—Dilutions										
		1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000	1–5000	1–6000
37.5° C.	17 hours	+	+	+	+	+	—	—	—	—	—	—
	43 "	+	+	+	+	+	+	I	I	I	—	—
	67 "	+	+	+	+	+	+	+	+	+	+	—
40° C.	17 hours	+	+	+	+	+	I	I	I	—	—	—
	43 "	+	+	+	+	+	+	+	I	I	I	—
	67 "	+	+	+	+	+	+	+	+	+	+	+
46° C.	17 hours	+	+	+	+	I	I	—	—	—	—	—
	43 "	+	+	+	+	+	+	+	+	+	+	I
	67 "	+	+	+	+	+	+	+	+	+	+	+
48° C.	17 hours	+	+	+	+	I	I	I	—	—	—	—
	43 "	+	+	+	+	+	+	+	+	+	+	—
	67 "	+	+	+	+	+	+	+	+	+	+	+
54° C.	17 hours	+	+	+	I	I	I	I	—	—	—	—
	43 "	+	+	+	+	+	+	+	+	+	—	+
	67 "	+	+	+	+	+	+	+	+	+	—	+

Temperature of incubator	Time of observation	SERUM 3—Dilutions					
		1–25	1–50	1–100	1–250	1–500	1–1000
37.5° C.	17 hours	+	+	+	—	—	—
	43 "	+	+	+	—	—	—
	67 "	+	+	+	—	—	—
40° C.	17 hours	+	+	I	—	—	—
	43 "	+	+	+	I	—	—
	67 "	+	+	+	I	—	—
46° C.	17 hours	+	+	+	—	—	—
	43 "	+	+	+	I	—	—
	67 "	+	+	+	I	—	—

Table 4—Continued
Effect of Different Incubation Temperatures on the Rate and Extent of Agglutination of Bovine Sera

Temperature of incubator	Time of observation	SERUM 3—Dilutions 1–25	1–50	1–100	1–250	1–500	1–1000
48° C.	17 hours	+	+	I	—	—	—
	43 "	+	+	+	—	—	—
	67 "	+	+	+	I	—	—
54° C.	17 hours	+	+	+	—	—	—
	43 "	+	+	+	—	—	—
	67 "	+	+	+	—	—	—

Temperature of incubator	Time of observation	SERUM 1—Dilutions 1–25	1–50	1–100	1–250	1–500	1–1000	1–2000	1–3000	1–4000
37.5° C.	17 hours	+	+	+	+	I	—	—	—	—
	43 "	+	+	+	+	+	I	—	—	—
	67 "	+	+	+	+	+	I	—	—	—
40° C.	17 hours	+	+	+	+	I	I	—	—	—
	43 "	+	+	+	+	+	I	—	—	—
	67 "	+	+	+	+	+	I	—	—	—
46° C.	17 hours	+	+	+	+	I	—	—	—	—
	43 "	+	+	+	+	I	—	—	—	—
	67 "	+	+	+	+	+	I	—	—	—
48° C.	17 hours	+	+	+	+	I	—	—	—	—
	43 "	+	+	+	+	+	—	—	—	—
	67 "	+	+	+	+	+	I	—	—	—
54° C.	17 hours	+	+	+	+	—	—	—	—	—
	43 "	+	+	+	+	I	—	—	—	—
	67 "	+	+	+	+	+	I	—	—	—

Results

No consistent differences were observed in the rate or extent of agglutination in the tests of any of the sera. Fleming (1) has suggested that the increased rate of agglutination in tests that are incubated in a water-bath at 55°C. is due, in part, to convection currents in the serum-antigen mixtures, which are enhanced by partially submerging the test tubes in a water-bath. The slight differences in the recorded titres of sera in this experiment were within the limits of experimental error and, in fact, do not exceed the discrepancies reported found (3) attributable to errors in observation of agglutination tests.

These four series of experiments substantiated the findings of those previously reported, relative to the relation between incubation at 37.5°C. and the desirable time of final observation of agglutination tests. To sum up the evidence on this point, it might be said that none of the incubation procedures employed in these studies resulted in maximum titres of all of the sera at the end of 24 hours. That is,

some sera showed higher agglutination titres at observations subsequent to 24 hours, with all the incubation procedures employed.

The fifth series of experiments was designed for the purpose of determining the importance, from the standpoint of numbers, of this slow-agglutinating group of sera. As previously outlined, the only sera that assume any importance in this regard (from a practical standpoint in the dilutions usually employed in routine diagnosis) are those with a low to medium agglutinin content (titre 1:25 to 1:100) which do not rapidly attain their maximum titres with any of the incubative procedures compared. In this series of experiments, duplicate sets of agglutination tests of 90 sera of low to medium agglutinin content were prepared. These were selected by testing numerous sera. A 24-hour observation of tests incubated at 37.5°C. was made and the sera were selected the titre of which did not exceed 1:100 at this reading. Some of the sera, after being reset for the comparisons of incubation procedures of agglutination tests, showed titres higher than 1:100 at observations later than 24 hours. This resulted in some overlapping between this group of sera of low to medium agglutinin content and those of the group that were considered as of high agglutinin content. However, this only tended to emphasize the importance of final observations later than 24 hours. One series of tests of all of the sera was placed in a hot-air incubator at 37.5°C., and a second series of tests was incubated in a water-bath at 37.5°C. over night and then held at room temperature. Observations were made and the results recorded after approximately 24, 48, and 72 hours.

The serum-antigen dilutions were 1:25, 1:50, 1:100, 1:250, 1:500, and 1:1000, using the polyvalent stock antigen (0.04% bacteria by centrifuge tube method).

Table 5 gives typical results of tests of 15 sera in this series of experiments.

Table 5

Results of Agglutination Tests of Low to Medium Agglutinin Content Sera (titre, 1:25 to 1:100) with Different Procedures of Incubation of Tests

Serum	Approximate time of observation	Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000											
		Hot-air incubator at 37.5° C.						Water-bath at 37.5° C. Overnight—room temperature					
31	24 hours	+	+	+	−	−	−	+	+	I	−	−	−
	48 "	+	+	+	−	−	−	+	+	I	−	−	−
	72 "	+	+	+	−	−	−	+	+	I	−	−	−
32	24 hours	+	+	+	−	−	−	+	+	+	−	−	−
	48 "	+	+	+	I	−	−	+	+	+	+	−	−
	72 "	+	+	+	+	−	−	+	+	+	+	I	−
6	24 hours	+	+	+	−	−	−	+	+	+	−	−	−
	48 "	+	+	+	+	−	−	+	+	+	I	−	−
	72 "	+	+	+	+	+	I	+	+	+	+	+	−

Table 5—Continued
Results of Agglutination Tests of Low to Medium Agglutinin Content Sera (titre), 1:25 to 1:100) with Different Procedures of Incubation of Tests

Serum	Approximate time of observation	Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000											
		Hot-air incubator at 37.5° C.						Water-bath at 37.5° C. Overnight—room temperature					
66	24 hours	+	I	—	—	—	—	+	I	—	—	—	—
	48 "	+	I	—	—	—	—	+	I	—	—	—	—
	72 "	+	I	—	—	—	—	+	I	—	—	—	—
67	24 hours	+	+	+	—	—	—	+	+	I	—	—	—
	48 "	+	+	+	—	—	—	+	+	I	—	—	—
	72 "	+	+	+	—	—	—	+	+	I	—	—	—
68	24 hours	I	I	I	—	—	—	+	+	I	—	—	—
	48 "	+	+	+	—	—	—	+	+	I	—	—	—
	72 "	+	+	+	—	—	—	+	+	I	—	—	—
73	24 hours	+	+	—	—	—	—	+	+	I	—	—	—
	48 "	+	+	+	—	—	—	+	+	+	—	—	—
	72 "	+	+	+	—	—	—	+	+	+	—	—	—
40	24 hours	+	+	—	—	—	—	+	I	—	—	—	—
	48 "	+	+	—	—	—	—	+	I	I	—	—	—
	72 "	+	+	—	—	—	—	+	+	I	—	—	—
21	24 hours	+	I	I	—	—	—	+	+	—	—	—	—
	48 "	+	+	+	—	—	—	+	+	I	—	—	—
	72 "	+	+	+	—	—	—	+	+	+	—	—	—
12	24 hours	+	+	—	—	—	—	+	I	—	—	—	—
	48 "	+	I	—	—	—	—	+	I	—	—	—	—
	72 "	+	+	—	—	—	—	+	I	—	—	—	—
33	24 hours	+	+	I	—	—	—	+	+	I	—	—	—
	48 "	+	+	+	—	—	—	+	+	+	—	—	—
	72 "	+	+	+	—	—	—	+	+	+	—	—	—
38	24 hours	+	+	—	—	—	—	+	+	—	—	—	—
	48 "	+	+	I	—	—	—	+	+	—	—	—	—
	72 "	+	+	I	—	—	—	+	+	I	—	—	—
8	24 hours	+	+	I	—	—	—	+	+	I	—	—	—
	48 "	+	+	+	—	—	—	+	+	+	—	—	—
	72 "	+	+	+	+	I	—	+	+	+	+	I	—
3	24 hours	+	+	I	—	—	—	+	+	+	—	—	—
	48 "	+	+	+	—	—	—	+	+	+	—	—	—
	72 "	+	+	+	I	—	—	+	+	+	I	—	—
9	24 hours	I	—	—	—	—	—	+	—	—	—	—	—
	48 "	+	I	—	—	—	—	+	I	—	—	—	—
	72 "	+	+	I	—	—	—	+	+	I	—	—	—

Results

In considering the results of this experiment, one should constantly keep in mind the fact that this group of 90 sera of low to medium agglutinin content represent only approximately 5% of the total number of bovine samples as they have been received in this laboratory for routine diagnosis. On the average, this number of suspicious reactions to the test would be encountered in testing 1,800 samples. It should be further considered, however, that this group of 90 sera represent approximately 20% of all serum samples in which

agglutinins have been demonstrated. On the average, 90 such sera would be encountered in testing 450 samples containing agglutinins.

The number of sera that attained their maximum titres and consequent satisfactory final observation at the end of 24 hours was found to be 47 (52.2%). The same 47 sera gave these results with both procedures of incubation. This 52.2% may therefore be considered to represent the approximate number of rapidly agglutinating low to medium agglutinin content sera. The remaining 43 samples (47.7%) did not attain their maximum titres at the end of 24 hours, consequently the desirable final observation was at a later time. It follows that the disposition of about 2.3% of all cattle tested in routine diagnosis might be influenced by the time of final observation of tests incubated at 37.5°C. This would be one animal in approximately 46 that are tested in routine diagnosis (probability 0.021). However, if the total number of sera to which these 43 samples are compared is reduced (by eliminating the negative sera) to include only sera in which agglutinins were demonstrated, the importance of this group becomes very different. In this case, these 43 samples of sera may be considered as having been selected from a group of 450 that were not negative to the agglutination test. The 43 samples would then represent approximately 9.5% of the sera containing demonstrable agglutinins for *Bact. abortus* antigens. This means that the disposition of one in approximately ten animals (probably 0.105) that were not negative might be influenced by the time of final observation of agglutination tests incubated at 37.5°C. These figures certainly indicate that it is not advisable to disregard the maximum titre and consequent diagnosis of this percentage of animals in order to obtain final results of agglutination tests at the end of 24 hours.

Similar studies and analyses of results of agglutination tests were conducted with a smaller group of sera of low to medium agglutinin content that had been incubated in water-baths at 50° C. and 55° C. The results were very similar except that a somewhat smaller number of sera failed to attain their maximum titres at the end of 24 hours. While the group of sera included in such studies was too small to justify expressing the results in percentages, it was very evident that final observations at the end of 24 hours, even with tests incubated at these higher temperatures, were not satisfactory for all sera.

The advisability of selecting the second day (approximately 40 to 48 hours) as the desirable time of final observation of agglutination tests incubated at 37.5°C. has been stated as follows: (3) "Low and medium agglutinin content sera, usually had reached their maximum titre (after 48 hours). In rare instances a slightly higher titre was

recorded at a later reading, but in such cases, the increase in titre was small. High agglutinin content sera showed complete agglutination in all except the highest dilutions. This indicates that it is feasible and safe to make the final reading in routine diagnosis at the end of 48 hours."

In order to obtain more detailed data regarding the advisability of the second-day observation as compared to the third-day observation of agglutination tests incubated at 37.5°C., the data on the agglutination tests of the 90 sera of low to medium agglutinin content mentioned above were analyzed. It was found that 76 (84.4%) of the 90 sera had attained their maximum titres at the second-day observation of tests incubated at 37.5°C. The second-day observation would therefore be entirely satisfactory as the final observation of this group of tests. The remaining 14 (15.6%) of the sera showed a higher titre on the third-day observation than recorded for the second-day observation. In most cases this increase in titre after the second day was very minor and would not have influenced the diagnosis and consequent disposition of the animals. In a few instances the diagnosis, derived from a fixed standard relative to agglutination in the various dilutions, would have been changed. It is, of course, realized that the diagnosis in animals having sera with a low to medium agglutinin content is sometimes influenced by factors other than the agglutination titre of a single test of the sera. These factors, can not, however, be considered in this statistical study of the results of agglutination tests. These 14 sera would represent only 0.7% of the calculated total of 1,800 samples of sera as they were received for routine diagnosis, which would be the equivalent of 1 in about 128 (probability 0.007) routine agglutination tests. Comparing the 14 sera with the calculated total of 450 samples containing demonstrable agglutinins, they represent 3.1 % or 1 in approximately 33 (probability 0.030) samples of agglutinating sera. It would be mathematical folly to express in percentages the number of these 14 sera in which this increase in agglutination titre after the second-day observation resulted in a change in the diagnosis. This, of course, is because the number of sera in the group is much too small for reliable mathematical analysis. However, it should be remembered that a change in diagnosis as a result of the third-day observation of the agglutination tests occurred only in a part of these 14 sera. From the practical standpoint of diagnosis in routine tests, therefore, the third-day observation would be necessary in only a part of 0.7% of all the tests, or in a part of 3.1% of tests of sera containing agglutinins. It appears from these results that the most satisfactory time of final observation of agglutination tests incubated at 37.5°C. is the third day, at approximately 72 hours. However, it seems that the discrepancy

between the observations on the second and third days is not of sufficient magnitude to justify discarding the second-day observation.

Similar analyses were made of the results of agglutination tests incubated in water-baths at 50° and 55° C. The numbers of sera of low to medium agglutinin ocntent in the groups were too small to be expressed in percentages. In these studies similar results were obtained except that there were relatively fewer sera in which the titre was higher on the third day than on the second day.

Summary

The statement quoted at the beginning of this paper, namely "It (agglutination) occurs more rapidly as the temperature rises up to an optimum of 55° C." was true in these experiments when the higher temperatures were obtained by partially submerging the test tubes in a water-bath. It was not apparent in tests incubated between 37.5° C. and 54° C. in hot-air incubators. The following statements are considered more comprehensive and correct to sum up the matter of the relation of temperature to rate of agglutination and the desirable interval before final observation of agglutination tests. (1) The rate of agglutination usually increases as the temperature rises up to an optimum of 55°C. (2) Some slow-agglutinating sera are encountered in all of the ranges of agglutinin content of sera. (3) With such sera, increasing the temperature of incubation of tests can not be substituted for the 48- to 72-hour time element necessary for attaining the maximum agglutination titres.

The following interpretations of the results of these experiments seem justified:

For purposes of research with the agglutination phenomonon, the final observation of agglutination tests should not be made until the third day, if any of the incubation procedures compared in these experiments are employed. In studies of the maximum titre of sera of excetionally high agglutinin content, the final observation should be on the fourth day.

For purposes of routine diagnosis with any of the incubation procedures employed in these experiments, the final observation should not be made until the second day (approximately 40 to 48 hours) when kept at 37.5°C. or above. Tests held at room temperature should not be finally recorded until 72 hours. If there is any doubt regarding the diagnosis, it is desirable to wait until the third day before the final observation is made of tests held at any temperature.

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XV. INFLUENCE OF HEMOLYSIS OF BOVINE BLOOD ON THE RESULTS OF THE TEST TUBE AGGLUTINATION TEST FOR BANG'S DISEASE

It is not uncommon for this laboratory to receive hemolyzed bovine blood for the diagnosis of Bang's disease. However, hemolyzed sera have not been sufficiently numerous to be considered a serious problem in the routine diagnosis of this disease. Consequently, it has not been considered necessary to recommend any drastic changes in the methods of collecting and transporting the specimens. This laboratory does not conduct the agglutination test of samples of sera that are badly hemolyzed. Other samples are received in which hemolysis is appreciable to the unaided eye, but is not extensive. Usually samples which only show slight hemolysis are accepted for the agglutination test and the results reported. It is not possible, with the unaided eye, to establish very definite standards for classifying hemolyzed samples into groups that are accepted or rejected for the agglutination test. In some cases, hemolyzed sera cause a noticeable discoloration of the serum—antigen mixture. This usually results in such tests being rejected at the time of observation. This, again, is a very indefinite means of differentiating the hemolyzed sera into groups that are or are not considered suitable for the test tube agglutination test. The results of tests of partially hemolyzed sera have usually been considered reliable if there were no changes in the antigen. That is, agglutination test (incubated at 37.5° C.) of hemolyzed sera which gave a negative reaction have been considered negative and the diagnosis was reported. On the other hand, when tests of hemolyzed sera result in changes in the antigen (which may or may not have appeared similar to serum-agglutination of the bacteria) the tests have been rejected and no diagnosis was reported. The practice in this regard in at least one other laboratory in which agglutination tests are held for 72 hours at room temperature, are known to be opposite. That is, in testing hemolyzed sera the other workers consider the results of a test positive if the antigen is agglutinated and return this diagnosis. Likewise, if there is no change in the antigen in the presence of hemolyzed serum, the tests are rejected and no diagnosis is reported.

With these differences in practices and opinions in mind, a series of experiments has been conducted with the idea of clearing up the

matter and learning what the correct procedure should be in handling hemolyzed sera for the test tube agglutination test for Bang's disease.

For this series of experiments, hemolysis in sera was purposely produced in two ways: (1) By freezing and thawing the blood after it had been collected and before the serum had been separated from the clot. (2) By adding distilled water to the blood at the time it was collected. The results of agglutination tests of sera containing hemoglobin would likely be uniform regardless of what forces liberated the hemoglobin from the red blood cells, except where chemicals, which would interfere with the agglutination reaction, had been added to the blood. Results similar to those reported subsequently were obtained in tests of sera in which there was visible hemolysis resulting from decomposition of the blood clot in tubes of serum held on the clot for long periods of time at room temperature.

A group of 17 animals whose sera had been repeatedly tested and found to represent a wide range of agglutinin content and a group of 25 animals with no agglutinins for *Bact. abortus* antigens were used to supply the sera.

In the first experiments, 7 separate sets of blood samples from individual animals in the foregoing groups were collected at different times and frozen to produce hemolysis. The number of sera in the series varied from 13 to 42, and the total number of hemolyzed samples tested was 166. Hemolysis of the blood was also produced by adding distilled water to the blood of three groups, with a total of 54 samples. Each of the groups of sera contained some samples having no agglutinin content and others in which there was a wide range of agglutinin content. Duplicate agglutination tests were prepared of each of the hemolyzed sera. One set was incubated at 37.5°C.; the other held at room temperature. The titres of the sera were always obtained by testing non-hemolyzed samples that had been collected at the same time. Observations of the tests were made and the results recorded after approximately 24, 48, and 72 hours. After 3 to 5 days at room temperature the tests were transferrred to the incubator and held for another three days.

The results of these experiments indicated that it was necessary to develop a method of producing different degrees of hemolysis in several samples of the same sera. This was accomplished by varying the amount of distilled water added to the blood at the time of collecting it from the animals. It was further necessary to estimate the quantity of hemoglobin in the hemolyzed sera. The Newcomer method (1) of the determination of hemoglobin in blood was utilized to give an estimation of the degree of hemolysis. Various amounts of serum were diluted with 10ml N/10 HCl. It was necessary to use different

serum dilutions because the degree of hemolysis varied and it was essential to have a concentration of acid hematin that could be compared to the Newcomer standard glass plate in the Klett colorimeter.

The formula used in deriving the amount of hemoglobin in 100 ml of serum was the same as that for blood by the Newcomer method, namely: 10 ÷ reading of the unknown on the colorimeter scale x0.0337x dilution of the serum x a correction factor for the dilution of the blood with water. (This factor varied in accordance with the amount of water used.) No corrections were made for (1) the carotin content of the serum, and (2) the factor 0.0337, which is not that of bovine blood but of swine blood.

The figures quoted are estimations of the hemoglobin by this method. While they are not accurate, they do form a satisfactory basis for comparison of the degree of hemolysis in the sera.

In these experiments sixteen cattle negative to the agglutination test for Bang's disease were selected to supply the sera. It was advisable to use only sera that were negative to *Bact. abortus* antigens for this work. Consequently any agglutination of the bacteria in the antigen, when tested with these sera could be associated with the presence of hemoglobin in the serum-antigen mixtures, and could not be attributed to specific agglutinins in the sera. Six samples of each of the sera were collected. The blood was drawn into centrifuge tubes to which varying amounts of distilled water had been added. The percentage concentration of distilled water in each of the series of 6 tubes of blood was 0%, 4.1%, 8.3%, 16.6%, 33.3%, and 50%. The quantities of hemoglobin per 100 ml. of serum were determined in accordance with the method described, for all of the samples. The amounts of hemoglobin in the samples varied from none to 11.3 grams per 100 ml. of serum. Freezing of blood at —8.8°C. overnight resulted in amounts of hemoglobin in the serum that varied from approximately 3 to 7 grams per 100 ml. of serum. Blood samples frozen at —23°C. overnight showed amounts of hemoglobin in the serum varying from approximately 7.5 to 10.5 grams per 100 ml. of serum. Agglutination tests were prepared in triplicate, using stock polyvalent antigen (0.04% bacteria by centrifuge tube method, 0.5% phenol as preservative). The serum-antigen dilutions were 1-25, 1-50, 1-100, 1-250, 1-500, 1-1,000. The addition of distilled water to the blood was taken into consideration in preparing the serum-antigen dilutions for the agglutination tests. One series of tests was held at room temperature, one at 37.5°C., and one at 52°C. Observations were made at 24, 48, and 72 hours. After three to five days at room temperature, such tests were transferred to the incubator and held for three additional days.

Table 1 and Table 1a give typical results of agglutination tests of 11 hemolyzed sera containing agglutinins for *Bact. abortus* antigens. These sera were taken from animals noted in the first series of experiments.

Table 1

Typical Results* of Agglutination Tests of Positive Hemolyzed and Non-Hemolyzed Bovine Sera with Phenolized (0.5%) Bact. Abortus Antigen

	Incubation conditions			
	Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000—72-hour observations			
Serum No.	37.5° C.	37.5° C.	Room temperature	Held at room temperature 5 days—then at 37.5° C.
	Non-hemolyzed sample	Hemolyzed sample	Hemolyzed sample	Hemolyzed sample
86	+ + + + + +	+ + + + + +	+ + + + + +	+ + + + + +
130	+ + − − − −	+ + + + − −	I I − − − −	+ + + + − −
129	+ + − − − −	+ + + − − −	I I − − − −	+ + + − − −
143	+ + + + + I	+ + + + + −	+ + + + + −	+ + + + + −
146	+ + − − − −	+ + + + + −	+ + − − − −	+ + + + + −
133	+ + − − − −	+ + + + + −	+ I − − − −	+ + + + + −
147	+ + + − − −	+ + + + + −	+ + I − − −	+ + + + + −
148	+ + − − − −	+ + + + − −	+ − − − − −	+ + + + I −
533	+ + + + I −	+ + + + + −	+ + + + I −	+ + + + + −
538	+ + − − − −	+ + + + − −	+ + − − − −	+ + + + − −

* No attempt was made to differentiate the types of agglutination from different causes by using different symbols. Many times it would have been easy to differentiate the types of agglutination by the appearance of the clumps of agglutinated bacteria. At other times, especially when agglutination of the bacteria was the result of a combination of the agglutinating forces, it would have been impossible.

Table 2 gives typical results of agglutination tests of 2 hemolyzed sera having no agglutinins for *Bact. abortus* antigens.

Table 1—Continued

Typical Results of Agglutination Tests of Positive Hemolyzed and Non-Hemolyzed Bovine Sera with Phenolized (0.5%) Bact. Abortus Antigen

SERUM 73—Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000

Concentration of distilled water in blood	Grams of hemoglobin per 100 ml. of serum	Approximate time of observation	Incubation conditions																					
			37.5° C.						52° C.						Room temperature						Held at room temperature 5 days—then at 37.5° C.					
50%	7.0213	24 hours	−	+	+	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	I	−
		48 "	+	+	+	I	I	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	I	−
		72 "	+	+	+	I	I	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	I	−
33.3%	7.0233	24 hours	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	I	−
		48 "	+	+	+	+	I	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	I	−
		72 "	+	+	+	+	I	I	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	I	−
16.6%	2.6167	24 hours	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−
		48 "	+	+	+	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−
		72 "	+	+	+	I	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−
8.3%	0.4578	24 hours	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		48 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		72 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4.1%	0.2647	24 hours	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		48 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		72 "	I	−	−	−	−	−	I	−	−	−	−	−	−	−	−	−	−	−	I	−	−	−	−	−
0%	0	24 hours	+	−	−	−	−	−	+	I	−	−	−	−	+	−	−	−	−	−	+	+	−	−	−	−
Non-hemolized sample		48 "	+	+	−	−	−	−	+	+	−	−	−	−	+	I	−	−	−	−	+	+	−	−	−	−
		72 "	+	+	−	−	−	−	+	+	−	−	−	−	+	+	−	−	−	−	+	+	−	−	−	−

Table 2

Typical Results of Agglutination Tests of Negative Hemolyzed Bovine Sera with Phenolized (0.5%) Bact. Abortus Antigen

Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000

SERUM 40—Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000																								
Concentration of distilled water in blood	Grams of hemoglobin per 100 ml. of serum	Approximate time of observation	Incubation conditions																					
			37.5° C.						52° C.						Room temperature						Held at room temperature 5 days—then at 37.5° C.					
50%	9.5726	24 hours	−	−	+	−	−	−	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
		48 "	+	+	+	+	+	−	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
		72 "	+	+	+	+	+	I	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	I
33.3%	7.0233	24 hours	−	−	+	−	−	−	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
		48 "	+	+	+	+	I	+	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
		72 "	+	+	+	+	+	−	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
16.6%	3.4111	24 hours	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−
		48 "	+	+	+	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−
		72 "	+	+	+	+	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−
8.3%	0.7491	24 hours	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		48 "	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		72 "	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4.1%	0.1326	24 hours	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		48 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		72 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
0%	0	24 hours	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Non-hemolized sample		48 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		72 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 2—Continued

Typical Results of Agglutination Tests of Negative Hemolzed Bovine Sera with Phenolized (0.5%) Bact. Abortus Antigen

Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000

SERUM 117—Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000

Concentration of distilled water in blood	Grams of hemoglobin per 100 ml. of serum	Approximate time of observation	Incubation conditions																					
			37.5° C.						52° C.						Room temperature						Held at room temperature 5 days—then at 37.5° C.					
50%	10.0275	24 hours	−	−	−	−	−	−	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
		48 "	+	+	+	+	I	−	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	I
		72 "	+	+	+	+	I	I	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	I
33.3%	8.0213	24 hours	−	−	I	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	−
		48 "	+	+	+	+	+	−	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
		72 "	+	+	+	+	I	I	+	+	+	+	+	I	−	−	−	−	−	−	+	+	+	+	+	−
16.6%	1.5344	24 hours	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−
		48 "	+	+	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−
		72 "	+	+	+	I	−	−	+	+	+	+	I	−	−	−	−	−	−	−	+	+	+	I	−	−
8.3%	0.6899	24 hours	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	I	−	−	−	−	−
		48 "	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
		72 "	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
4.1%	0.1934	24 hours	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		48 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		72 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
0%	0	24 hours	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Non-hemolized sample		48 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
		72 "	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Results

In the discussion of these results the term "true agglutination" refers to agglutination of the bacteria by specific agglutinins for *Bact. abortus* antigens. The term "false agglutination" refers to agglutination of the bacteria associated with the incubation of tests of hemolyzed sera and phenolized (0.5%) *Bact. abortus* antigen.

Incubation (37.5°C. or 52°C.) of agglutination tests of badly hemolyzed bovine sera (with no agglutinins for *Bact. abortus* antigens) invariably resulted in partial to complete "false agglutination" of the bacteria in phenolized (0.5%) *Bact. abortus* antigens in dilutions up to 1:500 or 1:1,000.

The dilutions in which this "false agglutination" reaction was observed varied in ratio to the quantity of hemoglobin in the sera. That is, "false agglutination" occurred in higher dilutions in tests wth badly hemolyzed sera (approximately 4 to 11 grams of hemoglobin per 100 ml. of serum) than in tests with other samples of the same sera having a lesser hemoglobin content (approximately 0.8 to 3.5 grams of hemoglobin per 100 ml. of serum). That is, "false agglutination" titres in tests with samples of hemolyzed sera varied in ratio to the amount of hemoglobin in the sera. When the hemoglobin content of the sera was comparatively low (approximately 0.5 grams of hemoglobin per 100 ml. of serum), this phenomonon was not apparent. The minimum amount of hemoglobin in the serum that was attended by this "false agglutination" was sufficient to be easily discernible with the unaided eye on careful examination. It was not sufficient to cause a noticeable discoloration of the antigen in serum-antigen dilutions of 1:50 or above.

The rate of "false agglutination" was definitely influenced by variations in the incubation temperature of the tests. It was usually not apparent or only very slight at the end of 24 hours incubation of tests at 37.5°C. After 48 hours' incubation of tests at 37.5°C., the extent of this phenomonon was only slightly less than the maximum that was observed after approximately 72 hours. In tests incubated at 52°C., this reaction was near the maximum after approximately 3 hours.

The results with agglutination tests of hemolyzed sera containing agglutinins for *Bact. abortus* antigens were very similar to those described with hemolyzed sera that did not contain any specific agglutinins. In this case, both "true agglutination" and "false agglutination" occurred in the tests. Usually in tests incubated at 37.5° C. the "true agglutination" of the bacteria in the antigen was more rapid than the "false agglutination." Consequently tests of positive hemolyzed sera showed "true agglutination" after 24 hours' incubation at 37.5° C. In tests of sera with low agglutinin content and high hemoglo-

bin content, the maximum titres, after 48 to 72 hours' incubation, were increased above the maximum titres of non-hemolyzed samples. This increase in maximum titre was the result of "false agglutination." Thus, the presence of specific agglutinins in hemolyzed sera did not interfere with the "false agglutination" of the bacteria in the antigen. Also, hemoglobin in the serum did not obliterate the activity of the agglutinin in the serum.

Tests of hemolyzed sera, containing specific agglutinins, that were incubated at 52° C. showed "false agglutination" before the "true agglutination" reaction had had time to take place. Consequently the "true agglutination" titres of sera of low agglutinin content were completely masked and "false agglutination" was observed.

As stated before, tests of sera having a low hemoglobin content (approximately 0.5 grams or less hemoglobin per 100 ml. of serum) did not show any "false agglutination." However, agglutination tests of some sera with this hemoglobin content and a low specific agglutinin content (titre 1-25 to 1-50) were negative. In other words, the existing "true agglutination" low titre of the serum was masked in the test by the effects of the presence of amounts of hemoglobin in the serum that were too slight to result in "false agglutination." That is, the results of tests of some sera with titres of 1-25 to 1-50, were interpreted as negative when the serum sample was slightly hemolyzed. It is apparent, therefore, that incubation of agglutination tests (with 0.5% phenolized antigen) at 37.5°C. or 52°C. produced a "false agglutination" reaction in tests of badly hemolyzed sera and partially masked a "true agglutination" reaction in tests of some slightly hemolyzed sera.

The results of agglutination tests (with 0.5% phenolized antigen) of hemolyzed bovine sera held at room temperature were very different from the results reported above. In this case no "false agglutination" was observed in any of the tests of hemolyzed sera. Tests of badly hemolyzed sera that did not contain agglutinins for *Bact. abortus* antigen were invariably negative after 3 to 5 days at room temperature but after being transferred to the incubator, "false agglutination" occurred. Hemolyzed sera, containing agglutinins for *Bact. abortus* antigens, showed "true agglutination" of the antigen in tests held at room temperature for 72 hours. The titres in such tests were usually somewhat lower than in tests of non-hemolyzed samples of the same sera. Usually the maximum titre of a given agglutinating serum was reduced approximately 50% if the serum was badly hemolyzed, e.g., a serum with a titre of 1-500 reduced to 1-250, or a serum with a titre of 1-50 reduced to 1-25.

The holding of agglutination tests (with 0.5% phenolized antigen) at room temperature for 72 hours offers a suitable technic for testing high titre hemolyzed bovine sera. It should be kept in mind that results of tests of hemolyzed sera that do not give clearly positive results should not be reported.

Summary

1. The results of these experiments indicate that the incubation (37.5° C., or higher) of agglutination tests of hemolyzed sera with 0.5% phenolized antigen for the diagnosis of Bang's disease is undesirable and the results of the test are inaccurate. Incubation of such agglutination tests resulted in a "false agglutination" reaction in some tests and a masking of the "true agglutination" reaction in others, depending on the hemoglobin content and the agglutinin content of the sera.

2. They further indicate that the holding of agglutination tests with 0.5% phenolized antigen for the diagnosis of Bang's disease at room temperature for 72 hours offers a suitable technic for testing hemolyzed bovine sera (such hemolysis resulting from either freezing of the blood or the addition of distilled water to the blood), providing it is taken into consideration that tests of hemolyzed sera in which results are not clearly positive should be rejected as not satisfactory and no diagnosis made.

3. It is very difficult to eliminate all hemolyzed sera from those that are accepted for the agglutination test for Bang's disease. This constitutes an argument against the practice of incubating at 37:5° C. or higher, agglutination tests of 0.5% phenolized antigen, for this disease.

The results of these experiments suggested the following studies to determine the factors involved in the formation of this precipitate that has been referred to as 'false agglutination."

Six bovine sera were used that contained no agglutinins for *Bact. abortus* antigen. Hemolysis of the blood was produced by adding distilled water at the time of collection. Non-hemolyzed samples were obtained at the same time. Tests were prepared by using the hemolyzed and non-hemolyzed samples. The addition of distilled water to the hemolyzed sera was taken into consideration in preparing the serum dilutions in the various test fluids. The following test fluids were used:

Distilled water + 0.85% NaCl
" " + " " + 0.5% phenol
" " + " " + 0.8% "
" " + " " + 0.1% cresol
" " + " " + 0.2% "
" " + " " + 0.25% formalin (40% formaldehyde gas in water)
" " + " " + 1% " " " " " "
" " + " " + 10% " " " " " "

Fresh unpreserved *Bact. abortus* antigen (concentration of bacteria 0.04%)
Bact. abortus antigen + 0.5% phenol
" " " + 0.8% "
" " " + 0.1% cresol
" " " + 0.2% "
" " " + 0.25% formalin (40% formaldehyde gas in water)
" " " + 1% " " " " " "
" " " + 10% " " " " " "

Tests of the hemolyzed and non-hemolyzed samples were prepared, using 1 ml. amounts of each of the test fluids. The serum-test fluid dilutions were 1-25, 1-50, 1-100, 1-250, 1-500, 1-1000. Duplicate sets of tests were prepared of the hemolyzed and non-hemolyzed samples. One series of tests was held at room temperature and the other series was incubated at 37.5°C. Observations were made after 48 and 72 hours. The experiment was repeated, using the same 6 sera. The results were identical to the first. Table 3 gives the results with one serum.

Table 3

Results of Tests of Hemolyzed Serum with Various Test Fluids and Incubation Temperatures

SERUM 70—Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000
72-hour observations

Test fluid	Hemolyzed serum												Non-hemolyzed serum											
	Incubated at 37.5° C.						Room temperature						Incubated at 37.5° C.						Room temperature					
Distilled water + 0.85% NaCl	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" " + " " + 0.5% phenol	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" " + " " + 0.8% phenol	+	+	+	+	+	+	+	+	+	+	+	+	M	M	M	—	—	—	M	M	M	—	—	—
" " + " " + 0.1% cresol	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" " + " " + 0.2% cresol	+	+	+	+	+	+	+	+	+	+	M	M	M	M	M	—	—	—	M	M	M	—	—	—
" " + " " + 0.25% formalin (40% formaldehyde gas in water)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" water + 0.85% NaCl + 1% formalin (40% formaldehyde gas in water)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" water + 0.85% NaCl + 10% formalin (40% formaldehyde gas in water)	—	—	—	—	+	+	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	—	—
Fresh unpreserved antigen (concentration of bacteria 0.04%)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bact. abortus antigen + 0.5% phenol	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" " " + 0.8% phenol	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—
" " " + 0.1% cresol	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" " " + 0.2% cresol	+	+	+	+	+	+	+	+	M	M	M	M	M	M	M	M	M	M	—	—	—	—	—	—
" " " + 0.25% formalin (40% formaldehyde gas in water)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" " antigen + 1% formalin (40% formaldehyde gas in water)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
" " antigen + 10% formalin (40% formaldehyde gas in water)	—	—	—	—	+	+	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—

+ = true agglutination or false agglutination.
— = no agglutination.
M = milky appearance of test fluid.

Results

With Fluids not Containing Bacteria

No precipitate ("false agglutination") was observed in any of the tests of non-hemolyzed sera. A milky appearance of the test fluid in the dilutions containing the greater amounts of serum was observed in tests containing the greater amounts of phenol and cresol.

A precipitate ("false agglutination") was present in incubated tests of hemolyzed sera when the concentration of phenol was 0.5% or more and in incubated tests containing 0.1% or more cresol.

No precipitate ("false agglutination") was observed in tests of hemolyzed sera held at room temperature for 72 hours when the test fluids did not contain more than 0.5% phenol or more than 0.1% cresol. A precipitate was present in tests of hemolyzed sera held at room temperature for 72 hours when the test fluids contained 0.8% phenol or 0.2% cresol.

The precipitate was very similar in appearance to the usual macroscopic appearance of agglutinated clumps of *Bact. abortus*. In fact, tests of hemolyzed sera with no bacteria in the test fluid were observed that could not be differentiated by macroscopic examination from positive agglutination tests.

This type of precipitate was not observed in incubated tests of hemolyzed sera in which the test fluid contained 5% or less formalin (40% formaldehyde gas in water). In such tests a flaky material was observed in the tests of some sera in dilutions containing the greater amounts of hemolyzed serum. It is doubtful if this flaky material would ever be confused with agglutinated clumps of *Bact. abortus*. The precipitate mentioned was observed in tests of hemolyzed sera in the dilutions containing the lesser amounts of serum when the test fluid contained 10% formalin. This precipitate was present in tests held at room temperature, and was more pronounced in incubated tests.

With Bacterial Antigens Containing Preservatives

The results were the same as those reported with fluids not containing bacteria. The bacteria suspended in the antigen were usually carried down by the precipitate in tests in which the precipitate occurred. This resulted in a clear fluid above the material precipitated in the bottom of the tubes. Such changes in the antigen have been referred to as "false agglutination."

With Fresh Unpreserved Bacterial Antigens

No precipitate was observed.

Summary

1. The precipitate ("false agglutination") observed in tests of hemolyzed sera apparently resulted from a reaction between the preservative in the test fluid and the hemoglobin in the serum.

2. This precipitate was not observed in tests held at room temperature when the test fluid did not contain more than 0.5% phenol or 0.1% cresol. It was observed in incubated tests containing these amounts of preservative.

3. Tests containing 0.25% to 5% formalin did not show this precipitate. Such tests of some hemolyzed sera did contain a flaky material.

4. No precipitate was observed in tests of hemolyzed sera and fresh unpreserved *Bact. abortus* antigen.

Discussion

In a previous paper on "The Influence of Temperature on the Rate of Agglutination of *Bact. Abortus* Antigen in the Test Tube Agglutination Test for Bang's Disease," it was shown that the most suitable time for final observation of routine agglutination tests incubated at temperatures up to 55°C. is the third day (approximately 72 hours). However, it was also shown that the second day is acceptable for final observations of routine agglutination tests incubated at 37.5° C., or higher. It was further shown that the results of tests were essentially the same after 72 hours regardless of variations in incubation temperature from room temperature to 55°C. In this paper it has been shown that hemolyzed sera interfere with the correct interpretation of the agglutination reaction in antigens containing the usual amounts of phenol and cresol as preservatives regardless of variations in the incubation temperature from room temperature to 52°C. It has been further shown that this interference is much less serious and injurious to the results of tests of phenol- or cresol-preserved antigens if the tests are held at room temperature. The results of these experiments further indicate that hemolyzed sera do not interfere with the results of agglutination tests of fresh, unpreserved antigen and the interference is not serious in tests of formalin preserved antigens. However, previous experiments (2) brought out objections to the use of unpreserved and formalin preserved *Bact. abortus* antigens. Another factor that is of importance is the relative cost of holding agglutination tests in routine diagnosis work at room temperature as compared to the cost of providing facilities for incubation of the tests. The holding of tests at room temperature has the disadvantage of delaying for one day the final results of the test.

The importance of hemolysis in suspected sera can not be estimated, it is a variable factor depending entirely on the numbers of

hemolyzed sera accepted for diagnosis and the extent of hemolysis in the samples. It is likely that hemolysis in sera does not constitute a serious handicap to the results of agglutination tests in any reliable laboratory with any technic of incubation of tests. On the other hand, it is likely that the presence or absence of hemoglobin in negative serum has not uncommonly resulted in serious discrepancies in the results of successive tests (with antigens containing phenol or cresol) of a given agglutinating serum when such tests have been incubated.

It is further likely that the agglutination titre of some low agglutinin content sera has been masked because of hemoglobin in the sera.

Conclusions

1. Room temperature is more satisfactory than incubation temperatures for holding agglutination tests of hemolyzed bovine sera with antigens containing 0.5% phenol or 0.1% cresol.

2. Fresh unpreserved and 0.25% formalin-preserved antigens are more satisfactory than 0.5% phenol or 0.1% cresol-preserved antigens for incubated tests of hemolyzed bovine sera for the diagnosis of Bang's disease. However, this advantage is overshadowed by disadvantages of such antigens for routine testing of hemolyzed and non-hemolyzed bovine sera.

3. It appears that workers using 0.5% phenol or 0.1% cresol preserved *Bact. abortus* antigen must choose between the disadvantages of incubating agglutination tests of bovine sera, some of which contain hemoglobin, and the disadvantage of delaying the results until the third day when holding the agglutination tests at room temperature.

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XVI. EFFECT OF HIGH TEMPERATURES ON *BACT. ABORTUS* AGGLUTININS

Many laboratories follow the practice of incubating agglutination tests in a water-bath at temperatures up to 55°C. for various lengths of time. Inactivation of the sera by similar heating processes prior to preparing the serum-antigen dilutions is a common procedure.

Fleming (1) states that the following may be taken to sum up the matter of relation of temperature to agglutination: "It occurs more rapidly as the temperature rises up to an optimum of 55° C., above which the reaction is impaired and soon ceases." Many water-baths that are not specially constructed are subject to some fluctuation in temperature.

A study has been made of the effect of water-bath temperatures on specific *Bact. abortus* agglutinin in bovine sera. No precision equipment for extreme accuracy was available or considered necessary, as the sole object of these experiments was to learn the approximate temperature that interferes with the activity of this agglutinin. These experiments were carried out as accurately as possible with the usual equipment for maintaining temperatures of water-baths.

Two series of experiments were conducted. In the first experiment 5 bovine sera were used (2 high agglutinin content, titre 1:500 or above; 2 low to medium agglutinin content, titre 1:25 to 1:100, and one with no agglutinin content). These sera were heated for 30 minutes in a water-bath at different temperatures. Each was divided into 8 samples, and a sample of each was heated at each of the following temperatures: 50°C., 55°C., 58°C., 60°C., 62°C., 65°C., and 70°C. An unheated sample of each serum was used in the agglutination test as a check on the maximum agglutination titre.

In the second experiment 15 bovine sera were used (10 high agglutinin content, 4 low to medium agglutinin content, and one with no agglutinin content). Samples of each were heated in a water-bath for 30 minutes, 1 hour, and 4 hours, at 58°C., 60°C., and 62°C.

The same polyvalent antigen (0.04% bacteria by centrifuge tube method plus 0.5% phenol) was used in all agglutination tests. The technic of preparing the serum-antigen dilutions was the same as that previously described.

Table 1 gives typical results of agglutination tests with heated bovine sera and the polyvalent antigen.

Table 1

Results of Agglutination Tests with Bovine Sera Containing Bact. abortus Agglutinin that Had Been Heated at Various Temperatures

Sera No.	Not heated	Time heated	Heated at 56° C.	Heated at 60° C.	Heated at 62° C.
	Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000				
144	+ + + + + +	30 min.	+ + + + + +	+ + + + + +	− I + + + +
				A A A A	
		1 hour	I I + + + +	− + + + + −	− I + + + +
					A A A A
		4 hours	I I + + + +		− I I I I −
128	+ I − − − −	30 min.	+ I − − − −	+ − − − − −	− − − − − −
		1 hour	I − − − − −	− − − − − C	
		4 hours	I − − − − −	− − − − − C	− − − − − C
139	+ + + + + +	30 min.	+ + + + + +	I I I I I I	− − − − + +
					A A
		1 hour	I − − − + +	− − − − I I	− − − + + +
		4 hours	+ − − I I I	− − − − − −	− − − − − −
185	+ + + + + −	30 min.	+ + + + + −	+ + + + + −	+ + + + + −
				A A A	A A A
		1 hour	+ + + + I I	I I I − − −	+ + + − − −
				A A A A	A A A A A
		4 hours	+ + + + + −	I I I I − −	+ + + + I −
130	+ + I − − −	30 min.	+ + I − − −	+ + − − − −	+ I − − − −
				A A	
		1 hour	+ + + − − −	I I − − − −	+ − − − − −
		4 hours	+ I − − − −	− − − − − C	− − − − − C
138	+ + I − − −	30 min.	+ + I − − −	+ + I − − −	+ + − − − −
		1 hour	+ + I − − −	+ + − − − −	+ + − − − −
		4 hours	+ I − − − −	+ + − − − −	+ + − − − −
143	+ + + + + −	30 min.	+ + + + + −	+ + + + + +	I I + + + −
		1 hour	I + + + I −	− I + + + I	− − I + + −
		4 hours	− I + + I −	− − − − − −	− − − − − −
129	+ + I − − −	30 min.	+ + I − − −	+ I − − − −	+ I − − − −
				A	A
		1 hour	+ I − − − −	I − − − − −	+ − − − − −
		4 hours	+ I − − − −	− − − − − C	− − − − − C
21	− − − − − −	30 min.	− − − − − −	− − − − − −	− − − − − −
		1 hour	− − − − − −	− − − − − −	− − − − − −
		4 hours	− − − − − −	− − − − − C	− − − − − C
142	+ + + + + −	30 min.	+ + + + + −	+ + + + − −	+ + + + − −
				A A A A	A A A
		1 hour	I + + + − −	+ + + I − −	+ + + − − −
				A A A	A A A
		4 hours	+ + + I − −	+ I I − − −	+ I I − − −
189	+ + + + + I	30 min.	+ + + + + I	I + + + + −	I I + + + I
		1 hour	I + + + + −	− − I + + −	I I I I − −
				A A	A A A
		4 hours	I + + + + I	− I I + + −	I I I + + −
86	+ + + + + +	30 min.	+ + + + + +	I I I I + +	− − − + + +
		1 hour	+ − − + + +	− − − − I I	− − − − − C
		4 hours	+ I − − + +	− − − − − −	− − − − − −

\+ = Complete agglutination.
I = Incomplete agglutination.
− = No agglutination.
A = Atypical appearance of clumps of agglutinated bacteria.
C = Serum partially coagulated.

Table 1—Continued

Results of Agglutination Tests with Bovine Sera Containing Bact. abortus Agglutinin that Had Been Heated at Various Temperatures

Sera No.	Not heated	Time heated	Heated At 56° C.	Heated At 60° C.	Heated At 62° C.
		Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000			
533	+ + + + + I	30 min.	+ + + + + I	+ + + + I −	I + + + I −
		1 hour	+ + + + − −	− − I I − −	− − I I I −
		4 hours	+ + + I − −	− − − − − −	− − − − − C
413	I + + + + −	30 min.	I + + + + −	+ + + + + −	+ + + + I −
		1 hour	+ + + + I −	+ + + + I −	+ + + + − −
				A A A A A	A A
		4 hours	+ + + + I −	+ + + + I −	+ + + + + −
153	+ + + + + +	30 min.	+ + + + + +	− − − − − −	
		1 hour	+ + + + + I	− − − − − −	
		4 hours	+ + + + + I	− − − − − −	

+ = Complete agglutination.
I = Incomplete agglutination.
− = No agglutination.
A = Atypical appearance of clumps of agglutinated bacteria.
C = Serum partially coagulated.

Results

Sera Heated to 50°C., and 55°C.

There was no apparent alteration to the agglutination titre of any of the sera tested.

Sera Heated to 58° C.

Sera heated for 30 minutes showed no appreciable change in maximum agglutination titres. There was a marked inhibition of the agglulation reaction with some of the samples of sera that had been heated for one hour. Other sera withstood this temperature for one hour with no appreciable influence on the activity of the agglutinin in the sera. After 4 hours in the water-bath the results were essentially the same as after one hour.

Sera Heated to 60° C.

After 30 minutes in the water-bath some sera showed an inhibition of the activity of the agglutinin and other sera showed no appreciable change in titre when tested with *Bact. abortus* antigen.

After one hour there was marked inhibition of the agglutination reaction of all the agglutinating sera. Some sera were coagulated by this heat; others were not coagulated. After 4 hours a larger number of the sera were coagulated but not all. There was a more marked inhibition of the agglutination reaction of sera heated 4 hours than was apparent with sera heated one hour.

Sera Heated to 62° C.

The results were similar to those with sera heated at 60°C., except that the inhibition of the activity of the agglutinin was slightly more extensive.

Sera Heated at 65° C. for 30 Minutes

There was no agglutination when these sera were tested. Coagulation of the serum was complete in some of the samples but not in all of them.

Sera Heated at 70°C. for 30 Minutes

All sera were completely coagulated, making it impossible to prepare agglutination tests.

Summary

1. The temperature that interfered with the activity of *Bact. abortus* agglutinin in bovine serum varied somewhat in the different sera. In some sera the agglutinin was partially destroyed by heating at 58°C. for one hour.

2. The temperature necessary to cause coagulation of bovine serum varied with different sera, the minimum being 60°C. for one hour.

3. It seems that incubation of agglutination tests for the diagnosis of Bang's disease in animals in a water-bath at 55°C. is a satisfactory procedure providing the thermo-regulating mechanism of the water-bath is sufficiently accurate to prevent the temperature of the bath from reaching approximately 58°C. at any time.

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XVII INFLUENCE OF THE AMOUNT OF ANTIGEN AND METHOD OF OBSERVATION OF AGGLUTINATION ON THE RESULTS OF THE AGGLUTIONATION TEST

It has been shown that the errors in observation of agglutination can be an important source of discrepancy in the results of the agglutination test (loc. cit.). Two amounts of antigen (1 ml. and 2 ml.) are in common use in different laboratories. Likewise two methods of observation of agglutination tests are in use. (1. Holding the tubes in the hand and shaking, observing the clearing of the antigen plus the extent of clumping of the organisms. 2. Observing the tubes in racks and basing the reading of the tests largely on clearing of the antigen.)

These two methods were compared. Thirteen bovine sera were used (4 high-agglutinin-content titre, 1:500 or above; 8 low to medium titre, 1:25 to 1:100, and one no agglutinin content). Duplicate sam-

ples of the sera were used to make a total of 44 samples. Agglutination tests of each were prepared with one and two ml. amounts of antigen. The tests were observed by five different persons and each individual observed a separate series of 44 tests prepared in duplicate with one and two ml. amounts of antigen. The serum-antigen dilutions were the same in tests prepared in the two amounts of antigen, namely 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000. An attempt was made to eliminate all errors in the preparation of the serum-antigen dilutions. All procedures in conducting the tests were the same in both methods except the amounts of antigen and serum and the systems of observation.

Results

The results indicated that there is no preference in these two methods from the standpoint of uniformity of the results of observation of agglutination. They further indicated that, on the average, slightly higher titres resulted from observation of tests of sera of low to medium agglutinin content when the tests were held in the hands and shaken for observation. This difference, however, was not sufficient to be of significance.

XVIII. EFFECT OF VARIOUS STAINS IN *BACT. ABORTUS* ANTIGEN ON THE SERUM AGGLUTINATION REACTION AND THE VISIBILITY OF MACROSCOPIC AGGLUTINATION

The observation of macroscopic agglutination in individual test tubes has been shown to be an important source of discrepancy in the results of the test tube agglutination test for the diagnosis of Bang's disease. (1) It seems likely that this source of discrepancy in the results of the agglutination test might be partly overcome if a suitable method of staining the antigen could be found. If a staining method was available that would color the organisms in the bacterial suspension without staining the salt solution in which the bacteria are suspended, and without influencing the serum-agglutination reaction, the observation and interpretation of agglutination would probably be more satisfactory and more uniform than it is with unstained *Bact. abortus* antigen.

Stains Employed

Stains are commonly employed in the preparation of concentrated antigens for use in the rapid agglutination test for the diagnosis of Bang's disease.

Various concentrations of the following staining materials have been used in the preparation of *Bact. abortus* antigen.

Gentian violet (Certification No. EB 1)
Basic carbol fuchsin (Certifiation No. E. F. 2)
Methylene blue (Grübler)
Safranin (Grübler
Thionin (Grübler)
Picro-fuchsin (5% of a 1% aqueous solution acid fuchsin in saturated solution picric acid)
Gram's iodine
Methyl violet (Grübler)
Potassium dichromate
Acid fuchsin (Coleman and Bell-Andrade's Indicator)
Eosin Y (Schultz No. 587 Certification No. L E 4)
Sodium pyrogallate
Aqueous extract of black walnut hulls

Results

None of the materials named proved satisfactory for staining *Bact. abortus* antigen for use in the test tube agglutination test. Many of them did not appreciably influence the serum-agglutination reaction when present in antigen, but there was not a sufficient advantage in the observation of agglutination to justify their use in the routine preparation of antigen. None of these materials stained the organisms without staining the carbolized saline solution in which the bacteria are suspended.

Bibliography

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XIX. EFFECT OF A PARTIAL VACUUM ON THE SERUM AGGLUTINATION OF *BACT. ABORTUS* ANTIGEN

In a previous publication (1) it was shown that the agglutination titres of more than 500 bovine sera containing *Bact. abortus* agglutinins were not appreciably influenced when the test tubes used for conducting the agglutination test were stoppered with corks.

An experiment was conducted in which the serum-antigen mixtures were held in a partial vacuum in test tubes. The partial vacuum was established by heating the test tubes, drawing out the heated glass, and sealing them. This, of course, was done after the serum-antigen dilutions had been placed in the test tubes. Duplicate tests were conducted in the usual manner as a check on the maximum titres of the agglutinating sera. Seven bovine sera with high agglutinin content, titre 1:500 or above; 3 with low to medium agglutinin content, titre 1:25 to 1:100; and 1 with no agglutinin) were used. The technic of preparing the serum-antigen dilutions was the same as described in a previous publication (1).

Results

The agglutination reactions of bovine sera were not appreciably altered in the tests that were held in a partial vacuum as compared to duplicate tests in unstoppered test tubes.

Conclusion

1. The free circulation of air is not essential to the serum agglutination reaction of *Bact. abortus* antigen.

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1. Fitch, C. P., Donham, C. R., Bishop, Lucille, and Boyd, W. L. Studies of Test Tube Agglutination Test for the Diagnosis of Bang's Disease. Minn. Tech. Bull. 73 (1930).

XX. EFFECT ON THE RATE OF AGGLUTINATION OF SHAKING MIXTURES OF AGGLUTINATING SERA AND *BACT. ABORTUS* ANTIGEN WITH A SHAKING MACHINE

Fleming (1) states: "The agglutination reaction as we do it comprises two quite different processes—firstly the union of the agglutinin of the serum with the bacteria, and secondly the clumping of the bacteria. The first process, the union of agglutinin and the bacterium, takes place very rapidly, and that by far the greater portion of the time in the ordinary reaction is occupied by the sensitized bacteria coming together in clumps." This author's work was concerned chiefly with a study of factors influencing the rate of agglutination. He

did not work with agglutination tests of *Bact. abortus* antigens. With regard to agglutination tests which were placed in a shaking machine for one hour, he states: "A considerable number of experiments have been done and it seems clear that a difference of temperature between 18° C. and 55° C. has no influence on the rate of flocculation of the bacteria. There is, however, some difference when flocculation takes place at very low temperatures. It is well known that gentle motion accelerates the flocculation."

In our work, experiments were conducted to determine the influence of shaking agglutination tests for Bang's disease on the rate of agglutination. An electrically driven shaking machine was used, such as is used in conducting the Kahn precipitation test for syphilis. (2) Agglutination tests were prepared in triplicate. One series of tests was shaken one hour after standing five to ten minutes at room temperature. Another series of tests was placed in a water-bath at 37.5°C. for one hour and then placed in the shaking machine for one hour. Observation of the tests was made after one-half hour and again after one hour of shaking. The tests were then placed in the incubator at 37.5°C. and observations made after approximately 24, 48, and 72 hours. The third series of tests was incubated 72 hours at 37.5°C. to determine the titres of the agglutinating sera. Twenty-four bovine sera (9 high agglutinin content, titre: 1:250 or above; 13 low to medium agglutinin content, titre 1:25 to 1:100; and one with no agglutinins) were used. The experiment was repeated and gentle motion was applied to the agglutination tests. A shaking machine that could be regulated for slow speed was used. The agglutination tests were shaken for one hour. Observations were made after 15, 30, 45, and 60 minutes.

Results

The titres of the agglutinating sera were essentially negative when observed immediately after the tests were removed from the shaking machines. There was some agglutination at this time in the lowest dilutions of tests of exceptionally high agglutinin content sera.

Conclusion

Shaking of agglutination tests for the diagnosis of Bang's disease was not successful as a means of hastening the agglutination reaction to a point that would allow immediate final observation of the tests.

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XXI. INFLUENCE ON THE RESULTS OF AGGLUTINATION TESTS OF DIFFERENT CULTURE MEDIA USED FOR GROWING *BACT. ABORTUS* FOR THE PRODUCTION OF AGGLUTINATION ANTIGEN

Several different culture media are being employed by various workers for growing *Bact. abortus* for use in the production of agglutination antigens. This has been commonly referred to as a possible cause of some of the discrepancies noted in the results of agglutination tests conducted in different laboratories. Henry and Traum (1) state as follows: "For over ten years, our antigen has been grown on 2% glycerin, 1% dextrose agar. Most other laboratories use liver infusion agar. Although able to obtain satisfactory growths and suitable cells for agglutination fluid, we are prepared to change to the more generally used liver infusion agar."

A series of experiments has been conducted to study the influence on the results of agglutination tests of different culture media used for growing bacteria for the production of agglutination antigen. Ten strains of *Bact. abortus* were grown on 3 kinds of culture media as follows: (1) 10% horse serum infusion agar, (2) 2% glycerin agar, (3) liver infusion agar as described by Huddleson (1). The organisms were grown in a 10% CO_2 atmosphere and were transferred approximately once a month for eight months. Three monovalent antigens from the three kinds of media were prepared for each of the strains every time they were transferred. Agglutination tests were prepared with each of the antigens using 6 bovine sera (2 with high agglutinin content, titre 1:500 or above; 3 with low to medium agglutinin content, titre 1:25 to 1:100; and one with no agglutinin content). Observations of the tests were made after approximately 24, 48, and 72 hours to record the rate of agglutination and the maximum titres of the agglutinating sera.

Results

The sensitivity of the antigens prepared from cultures transferred on horse serum agar, glycerine agar, and liver infusion agar for approximately eight months was not appreciably altered.

Discussion

It seems that the results of this experiment are confirmed by the experiences of various workers using different types of media over a period of years for the growth of *Bact. abortus* for agglutination antigen. In other words, suitable culture media seems not to affect appreciably the agglutination properties of *Bact. abortus* antigens.

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XXII. EFFECT ON THE AGGLUTINATION TITRES OF STORING BOVINE SERA AT REFRIGERATOR AND ROOM TEMPERATURES

It is frequently convenient and necessary to conduct agglutination tests of bovine sera several days after the sera have been obtained from the animals.

Buck (1) carried on a series of experiments in which various preservatives were added to samples of sera; other samples did not contain a preservative. He states that his experiments were not sufficiently extensive to justify definite conclusions.

The following is a summary of his work: "Refrigeration is decidedly advantageous to room temperatures in causing sera to retain their agglutinating properties and general appearances. Immediate centrifuging of the samples and removal of serum from clots did not appear to be advantageous to permitting the clots to contract for from 18 to 24 hours at room temperature and then decanting the serum. Samples to which nothing was added but which were kept in the Frigidaire seem to be the most satisfactory after approximately two months interval. The experiments, are, however, regarded as having shown that samples of serum if held for two months or more unless under favorable conditions may show a reduced agglutinin titre."

In our work a study has been made of the keeping qualities of *Bact. abortus* agglutinin in bovine serum, without the addition of a preservative to the serum. Fifteen bovine sera were used (9 high agglutinin content, titre 1:500 or above, 5 low to medium agglutinin content, titre 1:25 to 1:100, and one with no agglutinin). The blood was drawn into clean, sterilized glass test tubes. The sera were all collected the same day and allowed to clot at room temperature, after which the clot was loosened, and the tubes centrifugalized. Four samples of each sera were obtained. Two samples of each were decanted from the tubes containing the clot; the other two were allowed to stand in the original tubes, with the coagulated blood. Two samples of each of the sera (one clear serum without the clot and one serum on the clot) were placed in a refrigerator at about 3.5°C. The other two samples of each of the sera were held at room temperature. All of the tubes containing serums were unstoppered after being centrifugalized. No special precautions were taken to collect the blood in an aseptic manner. A clean sterilized hypodermic needle was used to draw the blood. The same needle was used on all animals. It was washed after the bleeding of each animal by forcing ordinary clean tap water through it with a syringe. The cattle were reasonably clean and no treatment was applied to the skin before the needle was introduced into the jugular vein.

Agglutination tests of the sera were conducted, using a polyvalent *Bact. abortus* antigen. Six serum-antigen dilutions were used as follows: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000. The technic of preparing the serum-antigen dilutions was the same as that previously referred to. The tests of the sera that were held at room temperature were made at the following intervals of days: 1, 4, 8, 10, 11, 14, 15, 18, 22, 26, 30, 39, 45, and 52. The tests of the samples held in the refrigerator were carried out at the following intervals of days: 1, 11, 18, 23, 28, 32, 36, 40, 43, 47, 50, 58, and 65.

Table 1 gives the agglutination test results with the 4 samples of each of 7 of the sera in the experiment (4 high agglutinin content, titre 1:500 or above; 2 medium agglutinin content, titre 1:50 to 1:100; and one with no agglutinin). These are typical of all the data.

Table 1
Results of Agglutination Tests with Bovine Sera After Storage at Different Temperatures

Manner in which sera were stored					
Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000					
Room temperature			Refrigerator, 3.5° C.		
No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot	No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot
	Serum 533			Serum 533	
0	+ + + + + −	+ + + + + −	0	+ + + + + −	+ + + + + −
4	+ + + + + −	+ + + + + I	11	+ + + + + I	+ + + + − I
8	+ + + + + I	+ + + + + I	18	+ + + + + I	+ + + + + −
10	+ + + + + −	+ + + + I − H	23	+ + + + + −	+ + + + I −
11	+ + + + + −	+ + + + + I H H	28	+ + + + I −	+ + + + + −
14	+ + + + + −	I I + + + + H H	32	+ + + + I −	+ + + + + − H H H H
15	+ + + + + I	I I + + I − H H	36	+ + + + + −	+ + + + + − H H H H
18	+ + + + + −	− I + + + − H H H	40	+ + + + + I	+ + + + + + H H H H
22	+ + + + + −	I + + + + − H H H H H	43	+ + + + + +	+ + + + − − H H H H H H
26	+ + + + I −	I I + + + −	47	+ + + + + −	I I I + + +
30	+ + + + + −		50	+ + + + + I	
39	+ + + + + I		58	+ + + + + −	
45	+ + + + + −		65	+ + + + + −	
52	+ + + + + −				

+ = Complete agglutination.
I = Incomplete agglutination.
− = No agglutination.
H = Hemolyzed.

Table 1—Continued
Results of Agglutination Tests with Bovine Sera After Storage at Different Temperatures

Manner in which sera were stored

Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000

Room temperature			Refrigerator, 3 5° C.		
No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot	No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot
	Serum 153			Serum 153	
0	+ + + + + +	+ + + + + +	0	+ + + + + +	+ + + + + +
4	+ + + + + +	+ + + + + +	11	+ + + + + +	+ + + + + +
8	+ + + + + +	+ + + + + +	18	+ + + + + +	+ + + + + +
10	+ + + + + +	+ + + + + +	23	+ + + + + +	+ + + + + +
11	+ + + + + +	+ + + + + +	28	+ + + + + −	+ + + + + +
14	+ + + + + +	− − I + + + H	32	+ + + + + I	+ + + + + I H
15	+ + + + + +	I I I + + +	36	+ + + + + +	+ + + + + +
18	+ + + + + +	+ + + + + − H H	40	+ + + + + +	+ + + + + + H H H
22	+ + + + + +	+ + + + + + H H H	43	+ + + + + +	+ + + + + + H H H H H H
26	+ + + + + +	− + + + + + H H H H	47	+ + + + + +	+ + + + + +
30	+ + + + + +	+ + + + + +	50	+ + + + + +	
39	+ + + + + +		58	+ + + + + +	
45	+ + + + + +		65	+ + + + + +	
52	+ + + + + +				
	Serum 86			Serum 86	
0	+ + + + + +	+ + + + + I	0	+ + + + + +	+ + + + + +
4	+ + + + + +	+ + + + + I	11	+ + + + + +	+ + + + I I
8	+ + + + + +	+ + + + + +	18	+ + + + + +	+ + + + + +
10	+ + + + + +	+ + + + + +	23	+ + + + + +	+ + + + + +
11	+ + + + + +	+ + + + + +	28	+ + + + + +	+ + + + I I
14	+ + + + + +	I I I I I + H H	32	+ + + + + +	+ + + + + +
15	+ + + + + +	I I I I I + H H	36	+ + + + + +	+ + + + I I
16	+ + + + + +	I I + + I I H H	40	+ + + + + +	+ + + + I I H H
18	+ + + + + +	I I + + + + H H H	43	+ + + + + +	− + + − + + H H H H H H
22	I I + I I I	I I I I I I H H	47	+ + + + + I	+ + + + + +
26	− − I I + +	I I − I + −	50	+ + + + + +	
30	I I I I I +		58	+ + + + + +	
39	− I I − I +		65	+ + + + + +	
45	− − − − − +				
52	− − − − − −				

+ = Complete agglutination.
I = Incomplete agglutination.
− = No agglutination.
H = Hemolyzed.

Table 1—Continued
Results of Agglutination Tests with Bovine Sera After Storage at Different Temperatures

Manner in which sera were stored				
Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000				
Room temperature			Refrigerator, 3.5° C.	
No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot	No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot
	Serum 139			Serum 139	
0	+ + + + + +	+ + + + + +	0	+ + + + + +	+ + + + + +
4	+ + + + + +	+ + + + + +	11	+ + + + + +	+ + + + + +
8	+ + + + + +	+ + + + + +	18	+ + + + + +	+ + + + + +
10	+ + + + + +	+ + + + + +	23	+ + + + + +	+ + + + + +
11	+ + + + + +	+ + + + + +	28	+ + + + + +	+ + + + + +
14	+ + + + + +	+ + + + + +	32	+ + + + + +	+ + + + + +
15	+ + + + + +	− I + + + +	36	+ + + + + +	+ + + + + +
16	+ + + + + +	− − I I − − H H	40	+ + + + + +	+ + + + + − H H H
18	+ + + + + +	I I I I − − H H H	43	+ + + + + +	+ + + I I − H H H H H H
22	+ + + + I I	+ + + I − − H H H	47	+ + + + + +	+ + + + + +
26	+ + + + + +	I I I + − −	50	+ + + + + +	
30	+ + + + + +		58	+ + + + + +	
39	+ + + + + +		65	+ + + + + +	
45	+ + + + + +				
52	+ + + + + +				
	Serum 130			Serum 130	
0	+ + + − − −	+ + + − − −	0	+ + + − − −	+ + + − − −
4	+ + + − − −	+ + + − − −	11	+ + + − − −	+ + I − − −
8	+ + + − − −	+ + + I − − H H H	18	+ + + − − −	+ + I − − −
10	+ + + − − −	+ + + − − − H H H	23	+ + I − − −	+ + + − − −
11	+ + + − − −	+ + + I − − H H H H	28	+ + + − − −	+ + − − − −
14	+ + I − − −	+ + + + − − H H H H H	32	+ + I − − −	+ + I − − −
15	+ + + − − −	+ + + + I − H H H H	36	+ + I − − −	+ + + − − − H H H H
18	+ + + − − −	+ + + + − − H H H	40	+ + I − − −	+ + + I − − H H H
22	+ + + − − −	− I + − − − H H H	43	+ + + − − −	+ + I − − − H H H
26	+ + + − − −	+ + I − − − H H H	47	+ + + − − −	+ + + − − −
30	+ + + − − −	+ + I − − − H H H	50	+ + + − − −	
39	+ + + + − −	+ I I − − −	58	+ + I − − −	
45	+ + + − − −		65	+ + I − − −	
52	+ + I − − −				

+ = Complete agglutination.
I = Incomplete agglutination.
− = No agglutination.
H = Hemolyzed.

Table 1—Continued
Results of Agglutination Tests with Bovine Sera After Storage at Different Temperatures

Manner in which sera were stored					
Dilutions: 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000					
Room temperature			Refrigerator, 3.5° C.		
No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot	No. of days sera were stored	Clear serum removed from blood clot	Clear serum on the blood clot
	Serum 129			Serum 129	
0	+ I I — — —	+ + I — — —	0	+ I I — — —	+ + I — — —
4	+ + I — — —	+ + — — — —	11	+ + I — — —	+ + I — — —
8	+ + I — — —	+ + + — — —	18	+ + I — — —	+ + I — — —
10	+ + — — — —	+ + I — — —	23	+ + — — — —	I + I — — —
11	+ + I — — —	+ + I — — —	28	+ + + — — —	I I I — — —
		H H			
14	+ + I — — —	I I — — — —	32	+ I I — — —	+ I — — — —
		H H H			
15	+ + I — — —	I I I — — —	36	+ + — — — —	+ I — — — —
		H H H			H H
18	+ + I — — —	— I I — — —	40	+ + + — — —	+ + — — — —
					H H H
22	+ + I — — —		43	+ + I — — —	+ I I — — —
		H H H			H H H
26	+ + I — — —	I I I — — —	47	+ + + I — —	I + I — — —
		H H H			
30	+ + I — — —	+ I I — — —	50	+ + I — — —	
		H H H H H			
39	+ + + — — —	+ + + + + —	58	+ + I — — —	
45	+ + + — — —		65	+ + I — — —	
52	+ + I — — —				
	Serum 21			Serum 21	
0	— — — — — —	— — — — — —	0	— — — — — —	— — — — — —
4	— — — — — —	— — — — — —	11	— — — — — —	— — — — — —
8	— — — — — —	— — — — — —	18	— — — — — —	— — — — — —
10	— — — — — —	— — — — — —	23	— — — — — —	— — — — — —
11	— — — — — —	— — — — — —	32	— — — — — —	— — — — — —
14	— — — — — —	— — — — — —	36	— — — — — —	— — — — — —
15	— — — — — —	— — — — — —	40	— — — — — —	— — — — — —
18	— — — — — —	— — — — — —	43	— — — — — —	— — — — — —
22	— — — — — —	— — — — — —	47	— — — — — —	— — — — — —
		H			
26	— — — — — —	— — — — — —	50	— — — — — —	
30	— — — — — —		58	— — — — — —	
39	— — — — — —		65	— — — — — —	
45	— — — — — —				
52	— — — — — —				

+ = Complete agglutination.
I = Incomplete agglutination.
— = No agglutination.
H = Hemolyzed.

Results

The sera that were decanted from coagulated blood and held in clean tubes retained their agglutinating properties for decidedly longer periods

of time than the duplicate serum samples stored in the original tubes with the blood clots.

The sera that were held in the refrigerator retained their agglutinating properties much longer than duplicate samples stored at room temperatures. The results of tests of sera are given below according to the manner in which the sera were stored.

Room Temperature Without Blood Clot

One of the sera showed a decrease in its agglutination titre after 18 days. Other sera showed inhibition of the agglutination reaction after longer periods of time. Most of the sera retained their original agglutinating properties in these dilutions up to the end of the experiment, which was 52 days.

Room Temperature With Blood Clot

The samples became visibly hemolyzed at about 10 to 14 days. All of the samples soon became badly hemolyzed and showed some inhibition of the agglutination reaction.

Refrigerator Temperature Without Blood Clot

All the sera retained their original agglutination titres in these dilutions throughout the experiment, which lasted 65 days.

Refrigerator Temperature With Blood Clot

Visible hemolysis of the sera developed at about 36 to 40 days and there was some inhibition of the agglutination reaction after this interval.

Discussion

It is not expected that the results would be identical to those reported if the experiment is repeated. It is likely that the number and kinds of contaminating bacteria would influence the keeping qualities of the agglutinin in sera, particularly if held at room temperature. It appears, however, that the following practices would usually be acceptable.

Sera that are tested 3 to 5 days after they are drawn usually would not have to be held at refrigerator temperatures or the serum to be removed from the clot. Both of these practices would be desirable. However, our experiments have shown that the titres of agglutinating bovine sera are not materially inhibited by the common practice of transporting blood from the field to the laboratory by mail. An occasional shipment of bovine serum may arrive at the laboratory in unsuitable condition for satisfactory testing. It appears that such failures in shipments that have been enroute only 1 to 3 days are attributable to factors other than merely the temperature and the presence of the blood clot. It is believed that the shaking of the samples in transit may re-

sult in hemolysis of the blood and thus render the serum samples unsuitable for the agglutination test.

Sera that are to be transported long distances or held for several days before the agglutination test is conducted should be removed from the clot. If this is done the titres of the sera can be expected to remain unaltered if the sera are held at room temperature for approximately 2 weeks and a large majority of samples for much longer periods of time.

Sera that are collected and held at refrigerator temperatures after decanting the clear serum from the coagulated blood can be expected to remain unaltered in agglutinin content for one to 2 months, or longer. The addition of a preservative to bovine serum containing *Bact. abortus* agglutinins is usually unnecessary.

Summary

1. Refrigerator temperatures are decidedly advantageous for storing bovine serum as compared to room temperatures.

2. Removal of the clear serum from the coagulated blood and storing it in clean sterile test tubes is decidedly advantageous as compared to holding the serum on the clot.

Bibliography

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GENERAL SUMMARY

1. The titres of agglutinating sera were not appreciably influenced by a broad zone in pH values of *Bact. abortus* antigens, such variation in pH resulting from the addition of dilute solutions of HCl and NaOH to the antigen.

2. The reactions of agglutinating sera were not appreciably altered when tested with *Bact. abortus* antigens having concentrations of NaCl between approximately 0.25% and 8%.

3. The rate of agglutination usually increases as the temperature rises up to an optimum of 55°C. Some slow agglutinating sera are encountered in all of the ranges of agglutinin content of sera. With such sera, increasing the temperature of incubation of tests can not be substituted for the 48 to 72 hour time element necessary for attaining the maximum agglutination titres.

4. A precipitate ("false agglutination") was observed in incubated tests of hemolyzed sera with antigens containing 0.5% phenol or 0.1% cresol. The "true agglutination" titre of some low agglutinin

content sera was masked in incubated tests when such sera containing hemoglobin were tested with such antigens.

5. Room temperature is more satisfactory than incubation temperatures for holding agglutination tests of hemolyzed bovine sera with antigens containing 0.5% phenol or 0.1% cresol.

6. Fresh unpreserved antigens and those preserved with 0.25% formalin are more satisfactory than antigens preserved with 0.5% phenol or 0.1% cresol for incubated tests of hemolyzed bovine sera for the diagnosis of Bang's disease. This advantage is, however, overshadowed by disadvantages of such antigens.

7. It appears that workers using *Bact. abortus* antigens preserved with 0.5% phenol or 0.1% cresol must choose between the disadvantages of incubating agglutination tests of bovine sera, some of which contain hemoglobin, and the disadvantage of delaying the results until the third day, when holding the agglutination tests at room temperature.

8. The temperature that interfered with the activity of *Bact. abortus* agglutinin in bovine serum varied somewhat in different sera. In some sera the agglutinin was partially destroyed by heating at 58° C. for one hour. The incubation of agglutination tests of bovine sera at 55° C. is a satisfactory procedure providing the thermo-regulating mechanism of the water-bath is sufficiently accurate to prevent the temperature of the bath reaching approximately 58° C. at any time.

9. Two methods (1) holding the tubes in the hands and shaking; (2) observing the tubes in racks of observation of agglutination tests with 1 ml. and 2 ml. amounts of antigen were compared. There was no preference in these two methods from the standpoint of uniformity of the results of observation of agglutination.

10. Shaking of agglutination tests (in shaking machines) for the diagnosis of Bang's disease was not successful as a means of hastening the agglutination reaction to a point that would permit immediate final observation of the tests.

11. The sensitivity of antigens prepared from cultures transferred on horse serum agar, glycerine agar, and liver infusion agar for approximately 8 months was not appreciably altered.

12. Refrigerator temperatures are decidedly advantageous for storing bovine serum as compared to room temperature. Removal of the clear serum from the coagulated blood and storing it in clean sterile test tubes is decidedly advantageous as compared to holding the serum on the clot.