STUDIES ON DISSOCIATION OF THE BRUCELLA GROUP

THESIS FOR THE DEGREE OF M. S.

Frank Anthony Gallo

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Michigan State College of Agriculture
and Applied Science

Studies on Dissociation of the Brucella Group

A Thesis
Submitted to the Graduate Faculty
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Department of Bacteriology and Hygiene
by

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Contents

Studies on Dissociation of the Brucella Group.

I - Introduction.

In recent years, the phenomenon of dissociation of bacteria has engaged the interest of many workers in the field of bacteriology. Although variability of bacteria was observed as early as 1875, the forms noted were regarded as contaminants rather than atypical forms. The relationship of the atypical forms was not recognized until recently.

Bacteriologists, accepting the fact that variation or dissociation does exist, are not attempting to ascertain whether the phenomenon is a haphazard "hit and miss" procedure or an orderly process.

Neisser and Massini (1906 - 1907) reported changes in Escherichia coli, which were quite significant. Kolle regarded their aberrant forms as contaminants, but Kawalenko (1910) confirmed their findings using single cell cultures. Their methods of study were quickly applied to other organisms and similar results were obtained. These studies acted as a stimulus to a more thorough study of the dissociation phenomenon. Among the many studies reported, may be cited the work of Cowan (1922) on the streptococci, Griffith (1928) on the pneumococcus, Topley and Ayerton (1924)

on the Salmonella enteritidis and Arkwright (1924) on Eberthella typhi. The repeated confirmation of dissociation of various organisms, by numerous workers lends convincing evidence that the phenomenon of dissociation is likely a property common to all bacterial species.

II - Historical Resume

The first studies on dissociation of the Brucella group were presented by Henry (1928 - 1929). He states that variants of the porcine strain, although culturally and morphologically similar to those of the bovine strain, appear to be widely separated serologically. Henry worked with two types of colonies, one type being the usual smooth Br. abortus colony described as being moist, clear, and slightly granular, and which by transmitted light shows a bluish green fluorescence. The second type of colony he describes as being opaque and granular. The organisms are suspended with difficulty in salt solution.

Frenzel (1931) was successful in dissociating Br. abortus to the R type. He states that the R strains are less virulent than the S types. His R antisera will not agglutinate the S antigen, but agglutinates the R antigen. The R types are weak when used as antigens, producing antisera of low titer.

On the other hand, he finds that the S antisera will agglutinate the R antigen in low dilutions.

In the light of the data which will be presented later, in this paper, the colony descriptions of Henry indicates that he was dealing with an intermediate or a partial R type of colony and not a true R type as he supposed.

Frenzel does not give a colonial description of the colonies, but points out agglutinative differences.

III - History of Cultures

The cultures in this work were obtained from the collection of Doctor I. F. Huddleson. The history of the cultures follows.

Brucella abortus.

Culture No. 1 was received from the Bureau of Animal Industry prior to 1915. The source and date of isolation is unknown.

Culture No. 2 was isolated from an aborted fetus in 1915, from herd "A" at Michigan State College.

Culture No. 3 was isolated from an aborted fetus in 1915, from herd "A" at Michigan State College.

Culture No. 4 was isolated from the udder of cow No. 995 of the abortion experimental herd of Michigan State College in 1915.

Culture No. 5 was in the laboratory stock cultures

prior to 1915. The source and date of isolation is unknown.

Brucella suis.

Culture No. 400 was obtained from Doctor Griswold of the Michigan Department of Health. It was isolated from a boar's testicle. The date of isolation is unknown.

Culture No. 401 was obtained from Purdue University. The source of the culture is not known. It was isolated January 15, 1925.

Culture No. 402 was obtained from Purdue University. The source and date of isolation is unknown.

Culture No. 404 was obtained from Doctor Conway? of the University of Missouri in 1922. It was isolated from a premature fetal pig, from a naturally infected sow, February 1, 1922.

Culture No. 405 was obtained from Doctor Conway? having been isolated from swine in Missouri. The date of isolation is unknown.

Culture No. 408 was obtained from Mr. Good of the University of Kentucky. It was isolated from a hog. The date of isolation is unknown.

Brucella melitensis.

Culture No. 301 was obtained from Mr. J. P. Torrey who isolated the culture from the Michigan State College Dairy herd. The date of isolation is unknown.

Culture No. 312 was isolated in 1921, from a case

of undulant fever in Tunis, Algeria by Doctor Burnet of the Pasteur Institute of Tunis.

Culture No. 315 was isolated from an undulant fever patient, by Doctor Burnet of the Pasteur Institute of Tunis.

Culture No. 316 is of human origin and was isolated by Doctor Burnet in 1928.

Culture No. 318 was received May 10, 1921 from Doctor K. F. Meyer of the George William Hooper Foundation, for medical research, University of California. The date of isolation is unknown.

IV - Bacteriological Study of Cultures.

Before dissociation studies were started, each organism was repeatedly plated out to eliminate all possible contamination. These strains were repeatedly stained by Gram's method to further check the purity of the cultures. The pure-line strains thus obtained were then studied culturally and physiologically to further check their identity.

The species of the strains selected for study were checked according to methods of identity presented by Huddleson, namely dye sensitivity and hydrogen sulphide production. Suffice it to state that Huddleson (1928 - 1929) found that the three species of Brucella can be recognized as measured by the source of the organism, namely bovine, porcine and caprine strains, by the agency of dye bacteriostasis

and hydrogen sulphide production as presented in the tables below.

TABLE I.

GROWTH ON DYE PLATES

	Thionin	Basic Fuchsin
Br. abortus	No growth	Growth
Br. suis	Growth	No growth
Br. melitensis	Growth	Growth

TABLE II.

PRODUCTION OF HYDROGEN SULPHIDE

	Hydrogen sulphide production in days.						
	1	2	3	4	5	6	7
Br. abortus	+	+	+	+	-	-	-
			-	-			
Br. suis	+	+	+	+	+	+	+
					-	-	-
Br. melitensis	-	-	-	-	-	-	-

The results presented in tables III and Plate I show that the strains selected for study checked with the identification given them by Doctor Huddleson.

The cultures were examined for their agglutinability by a Br. abortus immune serum obtained from an infected cow. The usual test tube method for agglutination was used. The results are presented in Table IV. It will be observed that all strains were

agglutinated in like titer although the maximum titer was not determined. However, the fact that all strains reacted similarly adds further proof for the identity of the cultures.

TABLE III.

BACTERIOSTATIC ACTION OF DYES ON ORGANISMS SELECTED FOR STUDY.

	Thionin	Fuchsin
Brucella Abortus (Bovine Species)		
1	No Growth	Growth
2	No Growth	Growth
3	No Growth	Growth
4	No Growth	Growth
5	No Growth	Growth
Brucella Suis (Porcine Strain)		
400	Growth	No Growth
404	Growth	No Growth
401	Growth	No Growth
405	Growth	No Growth
402	Growth	No Growth
408	Growth	No Growth
Brucella Melitensis (Caprine Strain)		
318	Growth	Growth
301	Growth	Growth
312	Growth	Growth
316	Growth	Growth
315	Growth	Growth

PRODUCTION OF H_2S
—BY—
SMOOTH STRAINS OF GENUS BRUCELLA

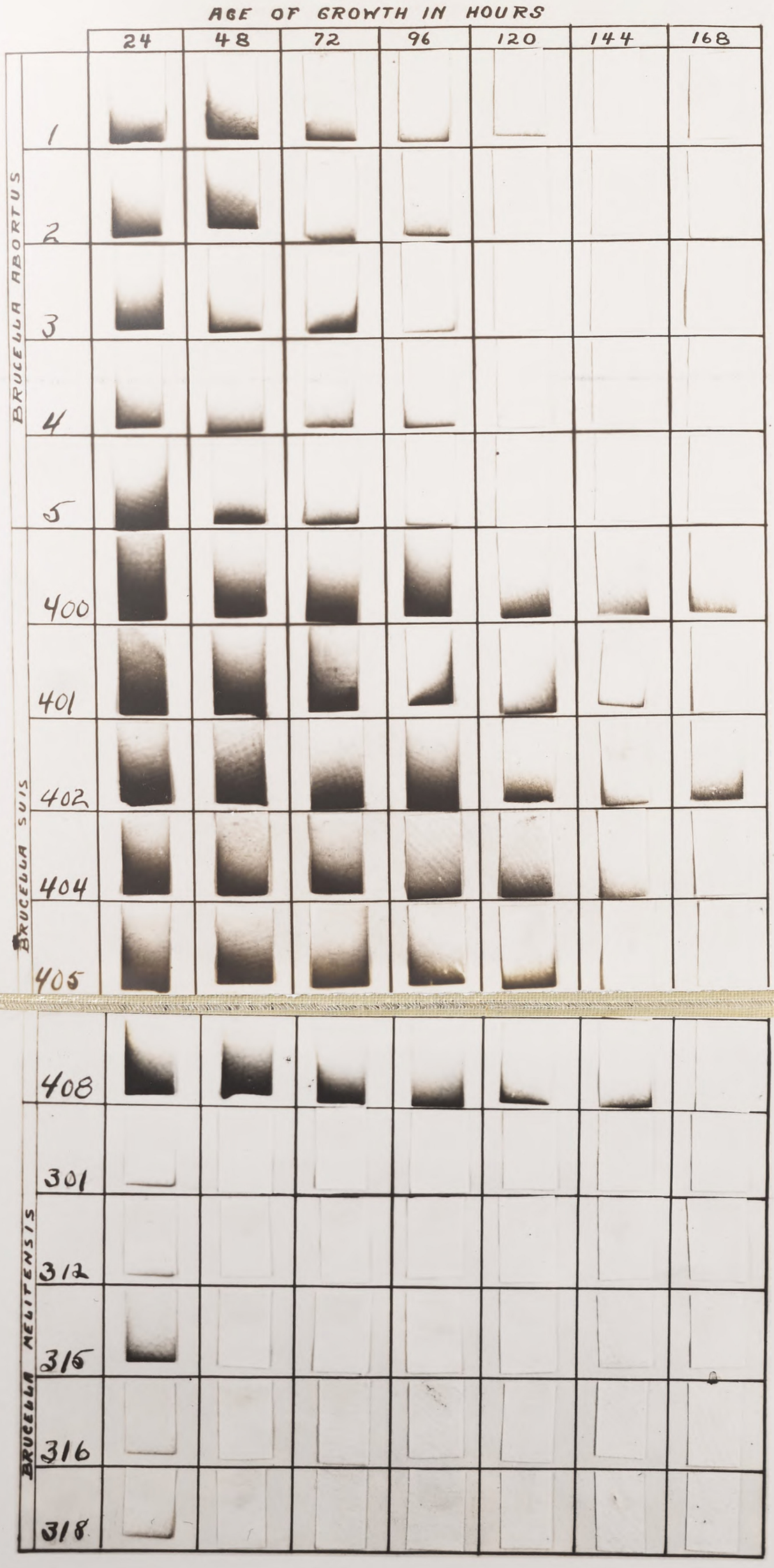

TABLE IV. THE AGGLUTINABILITY OF THE BRUCELLA ORGANISMS USED FOR DISSOCIATION.

	C	1-10	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	1-5000
Br. abortus											
1	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
2	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
3	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
4	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
5	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
Br. suis											
400	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
401	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
404	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
405	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
402	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
408	-	++++	++++	++++	++++	++++	++++	++++	+++	++	++
Br. meletensis											
316	-	++++	++++	++++	++++	++++	++++	++++	++	++	++
318	-	++++	++++	++++	++++	++++	++++	++++	++	++	++
315	-	++++	++++	++++	++++	++++	++++	++++	++	++	++
312	-	++++	++++	++++	++++	++++	++++	++++	++	++	++
301	-	++++	++++	++++	++++	++++	++++	++++	++	++	++

The sugar reactions were negative. The Brucella group, without exception, does not ferment the five sugars used. These reactions serve as a further check on the purity and identity of the cultures used.

The cultures selected for study were plated out repeatedly on liver infusion agar to study the colony formation. Without exception, the colonies were round and dome-shaped with a glistening surface. Under the low power of the microscope, the colonies gave a slightly granular appearance. The margins of the colonies were even and regular. From every standpoint all strains were typical S organisms.

The extreme care exercised in identifying and purifying the cultures selected for study is presented here to show that the cultures were pure-line strains of Brucella. This was done as a preliminary to the dissociation studies to rule out as effectively as possible the existence of contamination. Inasmuch as the R types of the Brucella were culturally, serologically, physiologically and morphologically different than the S prototypes, it is quite essential to know that the R forms described were not contaminants.

Beef liver infusion agar medium, first described by Stafseth (1920) and recommended by Huddleson (1927) was used through this work. This medium was selected

for this study, as Huddleson claims that it is the best medium for growing members of the Brucella group. The medium was prepared according to Huddleson's formula except that a Buchner funnel with a cotton filter was used, in the place of Scharples separator, for the clarification of the medium. Gentian violet was incorporated in the beef liver infusion medium, in a dilution of 1-50,000. The presence of this dye in the medium inhibits the growth of gram positive organisms. A more detailed description of the bacteriostatic action of this dye is given in an article published by Huddleson (1928).

Veal infusion broth was prepared in the usual manner, and adjusted to a ph of 7 in all instances. The one per cent lithium chloride broth was prepared by adding 10 cc. of a 10 per cent solution of lithium chloride to 100 cc. of veal infusion broth. The 0.1 per cent phenol veal infusion broth was prepared in a similar manner; 2 cc. of a 5.0 per cent solution of phenol was added to 98 cc. of veal infusion broth. The medium was then tubed and sterilized.

A high titered positive Br. abortus serum was used in the preparation of the positive serum broth. Ten cc. of sterile serum was added to 90 cc. of veal infusion broth. This was then tubed aseptically, incubated at 37°C. for a period of 24 hours, to eliminate contaminated tubes. The R immune serum

broth was prepared in a similar manner.

The test tubes used in serial transfer of the cultures were of standard height, having an internal diameter of one cm. This size tube was selected because it was found that the Brucella organisms can not be transferred serially by transferring a loopful of innoculum into 5 to 10 cc. of nutrient broth. Serial transfers can be obtained by transferring into 2 cc. or less of the broth. With this small amount of culture medium, the smaller diameter tubes were more satisfactory as evaporation was lessened by the smaller surface of the broth.

V - Experimental

I - Methods used to Bring About Active Microbic Dissociation.

Experiment No. 1

All strains of the Brucella group were seeded in 10 per cent positive serum broth, transferred serially and plated out every 48 hours. A series of these cultures were aged and plated out at weekly intervals. All plates were carefully examined for R type; colonies that showed rough colonial characteristics were fished and transferred on liver agar slants. The data are given in table V.

In 18 serial transfers of five strains of Br. abortus no R forms were observed. Opaque smooth forms

were observed in the eleventh transfer in all five strains studied. These persisted throughout the experiment with a complete disappearance of the S forms. The cultures that were transferred weekly showed similar results. In the case of Br. suis strain 408 produced R forms after the fifth transfer. The other five strains produced opaque-smooth forms after the eleventh transfer, but no R forms were observed.

The data presented in table VI shows the results of aging on dissociation of the species of Brucella. Opaque smooth forms were observed in Br. abortus at the fifth and sixth weekly transfer. Till then these cultures retained their original smooth characters. Strain 408 of Br. suis produced R type after the third weekly transfer; the remaining cultures retained their original smooth character until the last transfer, when they reverted to the smooth opaque forms. Strains 301 and 318 of Br. melitensis produced R forms after the fourth and fifth weekly transfer. The remaining cultures reverted to the opaque smooth, at the fourth transfer, going back to the S form at the fifth and sixth transfer.

TABLE V. DISSOCIATION CHANGES BY MEMBERS OF THE BRUCELLA GROUP INDUCED BY GROWING IN 10 PER CENT [illegible] SERUM BROTH.

Cultures	Time of Transferring																		
Br. abortus	4/30	5/2	5/4	5/6	5/8	5/10	5/12	5/14	5/16	5/18	5/20	5/22	5/24	5/26	5/28	5/30	6/2	6/4
1	-	-	S	S	S	S	S	-	S	S	OS	-	OS	OS	OS	OS	OS	-
2	S	S	S	S	S	S	S	S	S	S	OS	-	-	-	OS	OS	OS	OS
3	S	S	S	S	S	S	S	S	-	-	OS	OS	OS	OS	OS	OS	OS	OS
4	-	S	S	S	-	S	S	S	S	S	OS	OS	OS	OS	OS	OS	OS	OS
5	-	-	-	S	S	S	S	S	S	S	OS	OS	OS	OS	-	OS	-	-
Br. suis																		
400	-	S	S	S	S	S	S	S	S	S	OS	OS	OS	OS	OS	OS	-	OS
401	S	S	S	S	S	S	S	S	S	S	OS	OS	OS	OS	-	OS	-	OS
402	S	S	S	S	S	S	S	S	S	S	OS	OS	OS	OS	-	-	OS	OS
404	S	S	S	S	S	S	S	S	S	S	OS	OS	OS	OS	OS	-	OS	OS
405	S	S	-	-	S	S	S	S	S	S	OS	OS	OS	-	OS	OS	OS	OS
408	S	S	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R
Br. melitensis																		
301	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	R	R
315	S	S	-	S	S	S	S	S	S	S	S	OS	OS	OS	S	S	OS	OS
316	-	-	S	S	S	S	S	S	S	S	S	OS	OS	OS	S	S	OS	OS
312	S	S	S	S	S	S	S	S	S	S	-	OS	OS	OS	-	-	OS	OS
318	-	S	S	S	S	S	S	S	S	S	-	-	OS	OS	R	R	R	R

S = smooth type of colony
OS = smooth-opaque type of colony
R = rough type of colony
- = no growth

TABLE VI.

DISSOCIATION CHANGES BY MEMBERS OF BRUCELLA INDUCED BY GROWING IN 10 PER CENT SERUM BROTH.

Cultures						
Br. abortus	5/6	5/13	5/20	5/26	6/2	6/9
1	S	-	S	-	-	OS
2	S	S	S	S	OS	-
3	S	S	-	S	OS	OS
4	S	S	-	S	OS	OS
5	S	S	S	S	OS	OS
Br. suis						
400	S	S	-	S	S	-
401	S	-	S	S	S	OS
402	S	S	S	S	S	OS
404	S	S	S	S	S	OS
405	-	-	S	-	S	OS
408	S	S	S	R	R	R
Br. melitensis						
301	S	S	S	R	R	R
315	-	S	S	OS	S	S
316	S	S	S	OS	S	S
312	S	S	-	OS	S	S
318	S	S	-	OS	R	R

S = smooth type of colony
OS = opaque-smooth type of colony
R = rough type of colony
- = no growth

Of the Br. melitensis strains, culture 301 produced R forms after the eleventh transfer. Culture 318 produced R forms after the fifteenth transfer. The remaining three strains produced opaque-smooth types which persisted throughout the period of transferring.

Experiment No. 2

Tubes containing a one per cent solution of lithium chloride in veal infusion broth were inoculated with the cultures of Brucella. These cultures were transferred and plated out at weekly intervals for a period of ten weeks. All plates were examined for R types. The data are given in table VII.

From the data presented in table VII, it will be noted that no R forms of Br. abortus were obtained after ten weekly transfers. Opaque-smooth forms were observed in all cases after the third transfer. Strain 403 of Br. suis produced R forms after the fifth transfer. Opaque-smooth forms were observed in the remaining five strains after the fourth transfer. In the case of Br. melitensis, strain 318 produced R types after the seventh transfer. Opaque-smooth forms were observed in the remaining four strains after the fourth transfer. In all instances these opaque-smooth forms were quite stable and did not return to their original S forms.

Experiment No. 3

All cultures were transferred on liver infusion

agar slants, and aged for a period of six weeks. They were transferred on plain veal broth and plated out each week for a period of eight weeks. The data are given in table VIII.

III

TABLE ~~VII.~~ DISSOCIATION CHANGES BY MEMBERS OF THE BRUCELLA GROUP INDUCED BY GROWING IN ONE PER CENT LITHIUM CHLORIDE BROTH.

Cultures													
Br. abortus	5/6	5/13	5/20	5/28	6/6	6/11	6/18	6/25	7/2	7/9			
1			S	OS	OS	OS	OS	OS	OS				
2	S		S	OS	OS	OS	OS		OS	OS			
3	S	S	S	OS	OS		OS		OS	OS			
4	S	S	S	OS				OS	OS	OS			
5	S	S	S	OS				OS	OS	OS			
Br. suis													
400	S	S		S		OS	OS		OS	OS			
401	S	S	S	S		OS	OS		OS	OS			
402	S	S	S	S	OS	OS	OS	OS		OS			
404	S	S	S	S	OS		OS		OS				
405	S	S	S	S	OS		OS	OS	OS	OS			
408	S	S	S	S	R	R		R	R	R			
Br. melitensis													
301		S	S	S	OS	OS			OS	OS			
315	S	S	S	S	OS	OS			OS	OS			
316	S		S	S	OS	OS	OS		OS	OS			
312	S				OS	OS	OS	OS	OS	OS			
318	S		S	S	OS	SR		R	R	R			

S = smooth type of colony
OS = smooth-opaque type of colony
SR = smooth and rough type of colony
R = rough type of colony

IV

TABLE VIII. DISSOCIATION CHANGES BY MEMBERS OF BRUCELLA GROUP INDUCED BY AGAR OR LIVER AGAR SLANT THEN TRANSFERRED TO PLAIN BROTH.

Cultures	10/5	11/2	11/9	11/16	11/23	11/30	12/6	12/13
Br. abortus (Bovine strain)								
1	S	S	S	S	S	R	R	R
2	S	S		S	S	S	S	S
3		S	S	S	S	S	S	S
4	S			S	S		R	R
5	S	S	R	R		S	R	R
Br. suis (Bovine strain)								
401	S	S		S	S	S	S	S
403	S	S	S		S	R	R	R
402	S	S	S	S		R	R	R
404	S	R	R	R		R	R	R
405	S	S	S	S	S	S	R	R
408	S	R	R	R		R	R	R
Br. melitensis (Caprine strain)								
312	S	S			S	S	S	S
301	S	S	S	S	S	R	R	R
316	S	R	R	R			R	R
315		S	S	S	S	S	S	
318	S	S	S	S	S	R	R	R

S = smooth type of colony R = rough type of colony

Three out of the five strains of Br. abortus studied produced R forms. The R forms were observed as early as the third transfer and as late as the seventh transfer. In ten weekly transfers five out of the six strains of Br. suis studied produced R forms. Dissociation of this species was noted as early as the second transfer and as late as the sixth transfer. Strains 301, 316, 318 of the Br. melitensis cultures produced R forms after the first and fifth weekly transfers. The remaining cultures that did not dissociate retained their smoothness.

Experiment No. 4

The cultures were inoculated in tubes of veal broth containing 0.1 per cent phenol. They were transferred serially and plated at 48 hour intervals. All plates were carefully examined for R types. The data are given in table IX.

From the results indicated in table IX, it will be observed that all of the strains studied, retained their original smoothness after fourteen serial transfers. Strain 408 of Br. suis produced R types after the fifth transfer. It is interesting to note that strain 408 produced R types in each of the experiments that were performed to bring about dissociation. This organism was apparently quite unstable.

TABLE IX. DISSOCIATION CHANGES BY MEMBERS OF THE BRUCELLA GROUP INDUCED BY GROWING IN A 0.1 PER CENT PHENOL VEAL BROTH SOLUTION.

Cultures	9/30	10/2	10/4	10/6	10/8	10/10	10/12	10/14	10/16	10/18	10/20	10/22	10/24	10/26
Br. abortus (Bovine strain)														
1	S	S	S	S	S	S	S	S	S	S	S	S		S
2	S	S	S		S	S	S	S		S		S	S	S
3		S	S	S			S	S	S	S	S	S		
4	S		S	S	S	S		S	S	S	S		S	S
5	S			S	S	S	S			S	S	S	S	S
Br. suis (Porcine strain)														
401	S		S	S	S	S		S	S	S	S	S	S	S
400	S	S	S	S	S	S	S	S		S	S	S	S	S
402		S	S	S	S	S		S	S	S	S	S	S	S
404	S	S		S	S	S	S	S	S		S	S	S	
405	S	S		S	S	S	S	S	S	S	S		S	
408	S	S		S	R	R		R	R	R	R			
Br. melitensis (Caprine strain)														
312	S		S		S	S	S	S	S	S	S	S	S	S
301	S	S	S		S	S	S	S		S	S	S		S
316	S	S	S	S		S	S	S	S		S	S	S	S
315	S	S	S	S	S		S	S	S	S		S	S	S
318	S	S		S			S	S	S		S	S	S	S

S = smooth type of colony R = rough type colony

Growth of Cultures in 10 per cent CO_2.

The inoculated plates, in all experiments performed in this work, were placed in large glass jars, sealed and grown in an atmosphere containing 10 per cent carbon dioxide. The plates were then incubated at 37°C. Huddleson (1921) found that all the species of the Brucella group grew best in an atmosphere containing 10 per cent carbon dioxide.

There are a number of incitants used for microbic dissociation. In this work chemical agents, positive immune serum and aging were the means employed to bring about dissociation. All of the above mentioned agents have at some time been used successfully as dissociating incitants. The process of aging the cultures on liver agar slants, followed by weekly transfer on plain veal broth, gave the greatest amount of dissociation. The other agents used brought about dissociation in only a few of the strains that were under observation.

Biochemical studies of R strains.

All of the R strains were repeatedly plated on plain liver agar, and the typical R types were fished and transferred on liver agar. The action of these selected R types were studied on thionin and fuchsin. The dye plates were prepared as recommended by Huddleson (1923). The plates were seeded with heavy suspension of a 48-72 hour agar slant growth. The suspension was obtained by washing the growth from an

agar slant culture with a small amount of sterile broth. The seeded plates were then incubated at 37°C. for 48 hours. The data are given in table X.

From the data presented in table X, it will be noted that all R types of Brucella irrespective of their species grow equally well on both the thionin and fuchsin agar medium. It will be recalled that the smooth types of Br. abortus are inhibited in thionin medium, while the smooth types of Br. suis are inhibited on fuchsin medium. The R type of culture apparently develops a resistance or tolerance to the bacteriostatic action of thionin and fuchsin.

This experimental work with the R strains has been repeated several times to confirm the original reaction of these strains on the dyes and to strengthen the belief that these cultures that showed this particular character were not the results of contamination. The results in each instance were identically the same.

Fermentatively the R and S cultures do not differ. The R strains were transferred several times on sugars and each instance the results were identical.

Testing R Strains of the Brucella Group for Hydrogen Sulphide Production.

This method first described by Huddleson (1928) consists in the determination of hydrogen sulphide

TABLE X. THE RESULTS OF THE ACTION OF R FORMS OF THE BRUCELLA GROUP ON THE DYES THIONIN AND FUCHSIN.

Cultures	Thionin	Fuchsin
Br. abortus		
4	Growth	Growth
5	Growth	Growth
Br. suis		
400	Growth	Growth
405	Growth	Growth
402	Growth	Growth
404	Growth	Growth
408	Growth	Growth
Br. melitensis		
301	Growth	Growth
316	Growth	Growth
319	Growth	Growth

production over a definite period of time. The normal smooth cultures of Brucella produce varying amounts of hydrogen sulphide. Br. suis produces a considerable amount of hydrogen sulphide gas over a period of four days, Br. abortus produces a considerable amount of hydrogen sulphide gas over a period of two days, while Br. melitensis does not produce hydrogen sulphide gas. This, therefore, divides the species of this group into three classes, namely those that produce hydrogen sulphide (Br. suis) for a period of four days, those that produce hydrogen sulphide (Br. abortus) for a period of two days and lastly the strains of Br. melitensis that do not produce hydrogen sulphide.

Strips of lead acetate paper are placed inside the tubes beside the cotton plug, the paper extending slightly below the plug. The agar slants are heavily seeded previous with cultures to be studied. The paper is removed at 24 hour intervals and a fresh piece inserted in its place. This is repeated daily for seven days.

Experiment No. 5

The R strains were examined in a similar manner to determine their ability for hydrogen sulphide production. The data are presented in plate II. The R strains of Br. abortus did not produce hydrogen sulphide as did the homologous smooth strains. The R strains of Br. suis did not produce as much hydrogen sylphide as did the homologous smooth strains. The R strains of Brucella melitensis behaved the same as

their S prototypes.

Experiment No. 6.

Serological Studies on R Strains of the Brucella Group.

Rabbits were immunized using smooth and rough antigens of Brucella. Each of the animals received a series of five injections, which were given at weekly intervals.

Antigens of all the rough and smooth strains were made, the organisms being suspended in a 0.5 per cent phenolized physiological salt solution. Smooth antisera and R antisera were then obtained from the immunized animals. Cross-agglutination studies were then made on all of these various antigens using the different antisera. The data are presented in tables XI and XII.

PRODUCTION OF H_2S BY "R" TYPES OF GENUS BRUCELLA

AGE OF GROWTH IN HOURS

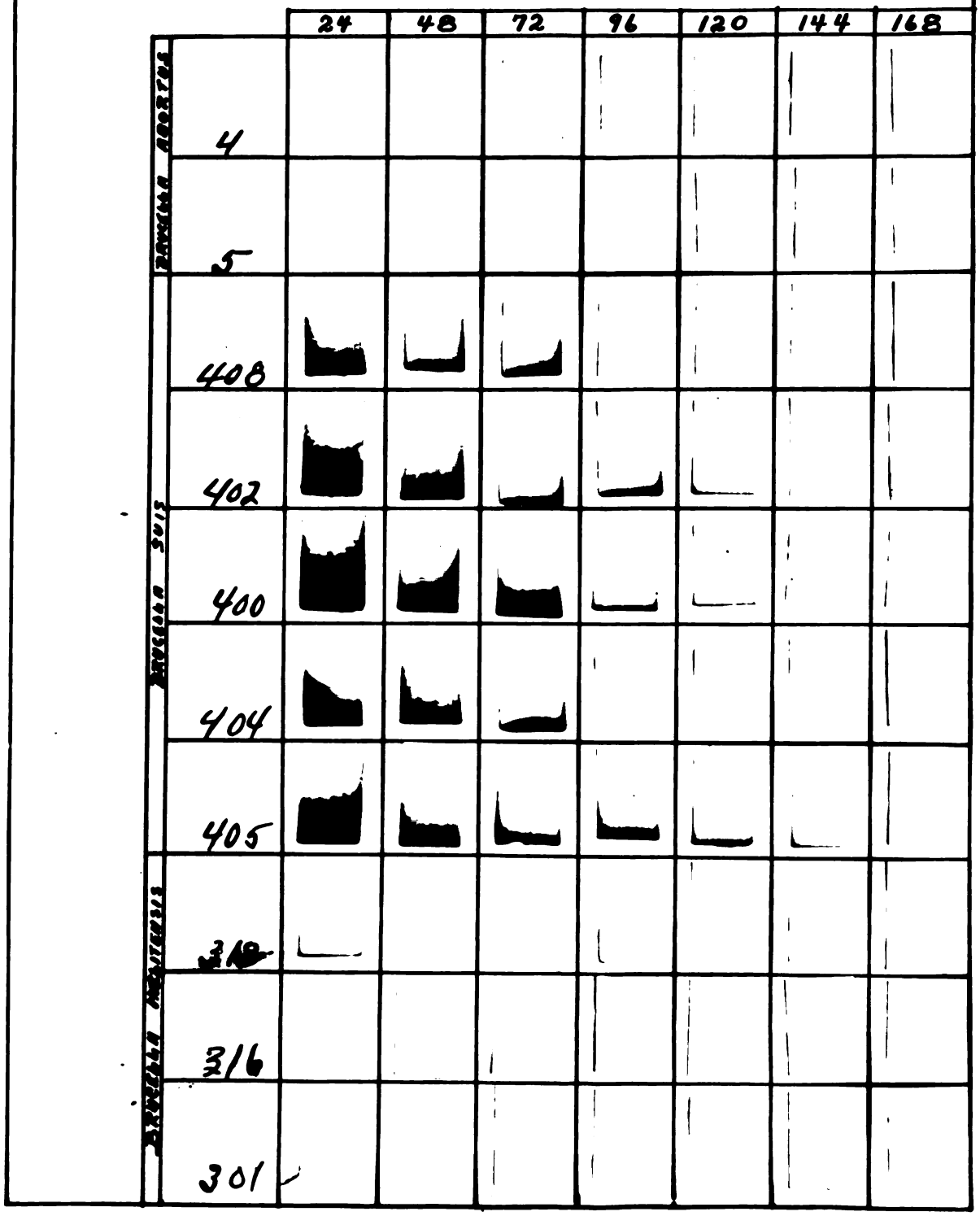

TABLE XI. AGGLUTINABILITY OF R STRAINS OF THE BRUCELLA ORGANISM USING R ANTISERA.

316 R Br. melitensis Antiserum	Serum dilutions										
Antigen	0	1-10	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	1-5120
316R	-	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
404R	-	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
5R	-	-	-	-	-	-	-	-	-	-	-
400R Br. suis Antiserum											
316R	-	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
400R	-	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
5R	-	-	-	-	-	-	-	-	-	-	-
4R Br. abortus antiserum											
316R	-	-	-	-	-	-	-	-	-	-	-
400R	-	-	-	-	-	-	-	-	-	-	-
5R	-	++++	++++	++++	++++	++++	++++	+++	+++	+++	++

300 series R Br. melitensis R type
400 series Br. suis R type
4 - 5 series Br. abortus R type

YL

TABLE XII. AGGLUTINABILITY OF R AND S STRAINS OF THE BRUCELLA GROUP USING NORMAL SMOOTH SERA OF THE RESPECTIVE SPECIES.

Culture #2 Br. abortus smooth serum — Antigens	Serum dilutions: 0	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	1-5120	1-10240
S	-	++++	++++	++++	++++	++++	++++	++++	++++	++++	++
S16	-	++++	++++	++++	++++	++++	++++	++++	++++	++++	++
400	-	-	-	-	-	-	-	-	-	-	-
5R	-	-	-	-	-	-	-	-	-	-	-
S16R	-	-	-	-	-	-	-	-	-	-	-
400R	-	-	-	-	-	-	-	-	-	-	-
Culture #316 Br. melitensis smooth serum											
S	-	++++	++++	++++	++++	++++	++	++	++	++	++
S16	-	+++	++++	++++	++++	++++	++++	++++	++++	+++	++
400	-	+++	++++	++++	++++	++++	+++	+++	+++	++	++
5R	-	-	-	-	-	-	-	-	-	-	-
S16R	-	-	-	-	-	-	-	-	-	-	-
400R	-	-	-	-	-	-	-	-	-	-	-
Culture #400 Br. suis smooth serum											
S	-	++++	++++	++++	++++	++++	++++	+++	+++	++	++
S16	-	++++	++++	++++	++++	++++	++++	+++	+++	+++	+++
400	-	-	-	-	-	-	-	-	-	-	-
5R	-	-	-	-	-	-	-	-	-	-	-
S16R	-	-	-	-	-	-	-	-	-	-	-
400R	-	-	-	-	-	-	-	-	-	-	-

R = rough

(1-5 series Br. abortus
300 series Br. melitensis
400 series Br. suis)

Rough strains of Br. suis and Br. melitensis show cross-agglutination, while the rough strains of Br. abortus will agglutinate only with its own specific R antiserum. There is no agglutination of the R antigens by the smooth serum, nor of the S antigens by the R antisera.

It will be observed from data presented in tables X and XI that the R antigens when inoculated into rabbits result in the formation of antibodies that will agglutinate the R but not the smooth antigen of the corresponding homologous strain. Inter-agglutinability of the R antigen occurs between the R Br. suis and R Br. melitensis, using the corresponding R homologous antisera. The rough abortus antigens are agglutinated by their own specific antiserum. These antigens will not be agglutinated by the antisera of either R Br. suis nor R Br. melitensis. Rough abortus antiserum will not agglutinate the R antigens of Br. suis and Br. melitensis.

The smooth antiserum does not agglutinate any of the rough antigens, irrespective of the antiserum used.

Morphology of R Cultures.

The R strains studied were characteristically different morphologically from their S prototypes. They differ both in size and shape. The R forms were without exception considerably larger than the S forms, sometimes being from 3 to 5 times larger. The organisms are long rods, very granular in structure and markedly

pleomorphic. Photomicrographs of R strains are presented on page 33. In all instances staining reactions with Gram's stain were similar to the original S forms.

Colonial Aspects of R Cultures.

The colonial appearance of the R type colony of Brucella is very similar in type to the R forms obtained in Salmonella group. The colonies are characterized by a very irregular contour, the edges being extremely jagged, resembling the usual soil spore-former colony. The surface of the colonies are wrinkled. The colony is flat with a dull appearance. The colonies have a brownish tinge, which becomes more marked with age. Photographs of smooth and rough colonies are presented on page 35.

Within the last ten years, many workers have at some time or other come in contact with organisms that gave rather peculiar and interesting reactions. Arkwright (1921) noted a variation in bacterial agglutination by the use of salts and specific serum. Griffith (1928) working with the pneumococci noticed the marked influence of immune serum on the biological properties of the organism. White (1925) made serological studies of the Salmonella group with the hopes of classifying them and he too observed the peculiar reaction of these strains to specific immune serum. This and the work of many others is too significant to be ignored, and to be looked upon as contamination.

Photomicrograph of R Types.

Figure 1.

Brucella abortus culture No. 4R

Magnification 756

Figure 2.

Brucella melitensis culture No. 516R

Magnification 970

Figure 3.

Brucella suis culture No. 404R

Magnification 756

Figure 1.

Figure 2.

Figure 3.

Photograph of Rough and Smooth Colonies.

Figure 4.

Brucella suis culture 402S

Magnification 460 ~~x~~

Figure 5.

Brucella melitensis culture 301R

Magnification 920 ~~x~~

Figure 6.

Brucella suis culture 402R

Magnification 460 ~~x~~

Figure 7.

Brucella abortus 4R

Magnification 460 ~~x~~

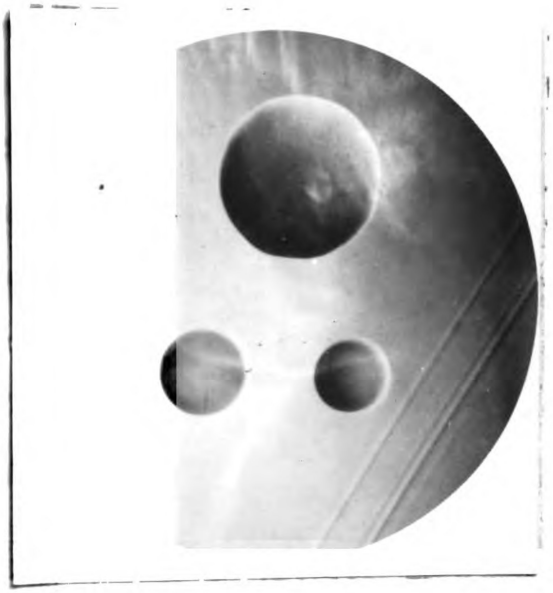

Figure 4.

Figure 5.

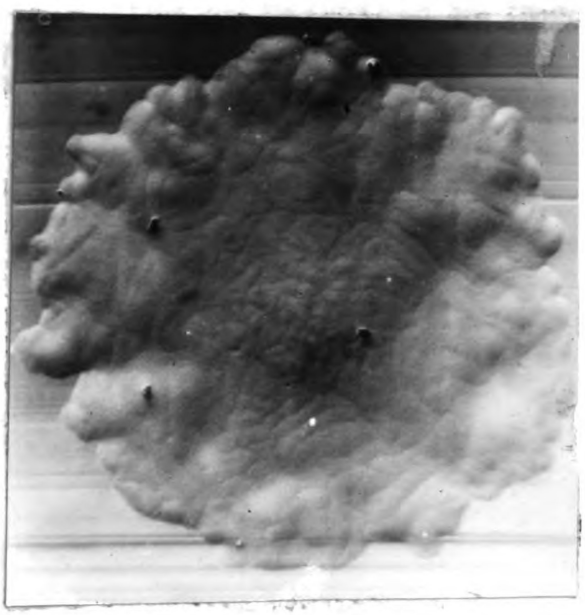

Figure 6.

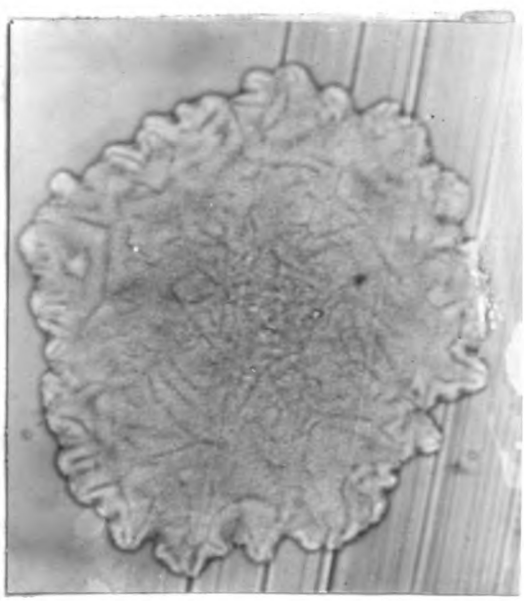

Figure 7.

The changes induced when a culture shifts from an S type to an R type are true variations. The changes induced are the results of shifts in cell character rather than the development of mixed strains. The microbic incitants merely cause the R character of the cell to develop to a greater extent than the S character with the result that a change in colony is obtained. The rate of the change may be quite gradual but generally it is of the latter type.

Reversion of R Types.

The R strains of Brucella were inoculated in 10 per cent R antiserum broth. A series of these strains were aged and transferred weekly. Another series was transferred serially every 48 hours. All strains were plated on plain liver infusion agar and gentian violet agar medium as described by Huddleson (1928). The data are presented in tables XIII, XIV, XV and XVI.

TABLE XIII. REVERSION OF R TYPES INDUCED BY GROWING THEM ON 10 PER CENT R ANTISERUM BROTH. (CULTURES WERE PLATED ON PLAIN LIVER AGAR.)

Cultures	Time of Incubation												
Br. abortus	2/23	2/25	2/27	3/1	3/3	3/5	3/7	3/9	3/11	3/13	3/15	3/17	
4R	R	R	R	R	R	-	R	R	-	R	R	R	
5R	R	R	R	-	R	OS	OS	S	S	S	S	S	
Br. suis													
2	R	R	R	R	RS	RS	-	R	R	R	-	-	
402R	R	R	R	R	R	R	RS	R	R	R	R	R	
403R	R	R	R	R	R	OS	OS	S	S	S	S	S	
404R	R	R	R	R	R	R	-	R	R	-	R	R	
405R	R	R	R	R	R	R	-	R	R	R	R	S	
Br. melitensis													
301R	R	R	R	R	R	R	-	-	R	R	R	R	
316R	R	R	R	R	R	R	R	R	-	-	R	R	

R = rough type of colony
S = smooth type of colony
OS = smooth-opaque type of colony

VIII

TABLE XIV. REVERSION OF R TYPES INDUCED BY AGING IN 10 PER CENT R ANTISERUM BROTH. (THESE AGING WERE PLATED ON PLAIN LIVER AGAR.)

Cultures	Time of Incubation					
	3/2	3/9	3/16	3/23	3/29	4/5
Br. abortus						
4R	R	R	R	R	R	R
5R	R	OS	S	S	S	S
Br. suis						
400R	R	-	R	R	R	R
402R	R	R	R	R	R	R
408R	R	OS	S	S	S	S
404R	R	R	R	R	R	R
405R	R	R	R	R	R	R
Br. melitensis						
301R	R	R	R	R	R	R
316	R	R	R	R	R	R

R = rough type of colony
S = smooth type of colony
OS = smooth-opaque type of colony

TABLE XV. REVERSION OF R TYPES INDUCED BY GROWING THEM ON 10 PER CENT R ANTISERUM BROTH. CULTURES WERE PLATED ON PLAIN LIVER INFUSION AGAR CONTAINING GENTIAN VIOLET (1 - 50,000).

Cultures														
Br. abortus	2/23	2/25	2/27	3/1	3/3	3/5	3/7	3/9	3/11	3/13	3/15	3/17		
4R	R	R	R	R	-	OS	S	S	S	S	S	S		
5R	R	R	-	OS	OS	S	S	S	S	S	S	S		
Br. suis														
400R	R	R	R	R	OS	OS	S	S	S	S	S	S		
402R	R	R	R	R	R	OS	OS	S	S	S	S	S		
408R	R	R	R	R	OS	OS	S	S	S	S	S	S		
404R	R	R	R	R	OS	OS	OS	S	S	S	S	S		
405R	R	R	R	R	RS	OS	OS	OS	OS	S	S	S		
Br. melitensis														
301R	R	R	R	R	R	R	R	R	R	R	R	R		
316	R	R	R	R	R	R	-	-	R	R	R	R		

R = rough type of colony
S = smooth type of colony
OS = smooth-opaque type of colony

TABLE XVI. REVERSION OF R TYPES INDUCED BY GROWING THEM ON 10 PER CENT R ANTISERUM BROTH. THESE AGING CULTURES WERE PLATED AT WEEKLY INTERVALS ON PLAIN LIVER AGAR CONTAINING GENTIAN VIOLET (1 - 50,000)

Cultures						
Br. abortus	3/2	3/9	3/16	3/23	3/29	4/5
4R	R	OS	S	S	S	S
5R	R	OS	S	S	S	S
Br. suis						
400R	R	OS	S	S	S	S
402R	R	OS	S	S	-	S
408R	R	OS	S	S	S	-
404R	R	OS	S	S	S	S
405R	R	R OS		S	S	S
Br. melitensis						
301R	R	R	R	R	R	R
316R	R	R	R	R	R	R

R = rough type of colony
S = smooth type of colony
OS = smooth-opaque type of colony

From the data presented in table 13, it will be observed that the reversion from the R to the S type was obtained in two instances. Br. abortus (culture No. 5R) and Br. suis (culture No. 408R) reverted to their homologous types. The R colony did not shift to the S form after twelve serial transfers. Table 14 presents data, showing the effect of aging the cultures. It will be noted, that the same strains referred to above reverted to their S forms after the second weekly transfer. The remaining strains retained their rough colonial appearances. It is interesting to note that these strains were plated on plain liver infusion agar. This type of medium favors the R forms, resulting in the appearance of R type colonies on the plate.

The data presented in table 15 demonstrated the reversion of the R types back to their homologous S forms. A shifting of the R colony to the S type was observed with all of the R strains of Br. abortus and Br. suis reverted to their homologous S forms. The R types of Br. melitensis retained the rough colonial characteristics. In this case, the cultures were plated out on liver infusion agar medium containing gentian violet in a dilution of 1-50,000. This type of medium favors the shifting of the R colony to the S forms, for a greater number of strains reverted to their homologous types when this medium was used. Reversion of R types

by animal inoculation with a suspension of S killed organisms and living R organisms.

Griffith (1928) was able to revert R types of pneumococci to their homologous S types by injecting the R organism together with the killed S organism subcutaneously into white mice. This work was confirmed by Dawson (1930).

This method of reversion was tried on R types of the Brucella cultures. The suspension of living and killed organisms were prepared in the usual manner. The suspensions of killed organisms were plated out to check sterility. Equal amounts of the living R organisms and killed S organisms were mixed together, diluted with sterile physiological salt solution so as to give a turbidity reading of 7 mm on the Gage Nephelometer. Living R suspensions were prepared in a similar manner.

Guinea pigs were inoculated subcutaneously with the mixtures of killed S and living R organisms. Guinea pigs were also inoculated with suspensions of living R organisms alone. A series of four injections were given the animals, at weekly intervals. The guinea pigs were killed and autopsied.

Table 17 gives the data concerning post-mortem results, tissues from which organisms were isolated,

action of isolated organisms on dyes, hydrogen sulphide production of isolated organisms and agglutination tests with smooth abortus anti-serum.

In the guinea pigs receiving the living R organisms and killed S organisms, S strains were isolated from the liver, spleen and lungs. S organisms were also isolated from the liver, spleen and lungs. S organisms were also isolated from guinea pigs that received living R organisms, although the organisms were not isolated from all tissues that were cultured.

Study of reverted strains

It will be noted that all R types of the Brucella group reverted to their smooth homologous strains. The R colonies reverted in all instances, to the S type by injecting subcutaneously suspension of killed S and living R organisms. These cultures reacted the same as did the normal smooth types, in regards to action on dye plate, hydrogen sulphide production and agglutination.

It will also be noted that none of the R types were stable roughs. The R types of Br. melitensis were the most stable, since they did not revert to their homologous smooth types by rapid transferring, but did revert on animal passage using a suspension of living R and killed S organisms.

TABLE XVII. AUTOPSY RESULTS, TISSUES FROM WHICH CULTURES WERE ISOLATED, ACTIONS OF ISOLATED CULTURES OR DYES, HYDROGEN SULPHIDE PRODUCTION AND AGGLUTINATION.

Guinea pig	Post mortem findings All tissues cultured	Cultures isolated from following tissues	Action of isolated cultures on dyes Thionin	Fuchsin	Hydrogen sulphide production, days 1 2 3 4 5 6 7	Agglutination test using abortus S serum 1-20	1-40	1-80	1-160	1-320	1-640
G. pig No.1 Vaccine 316R Bacterin 316S	Li. enlarged numerous gray-white foci. Sp. numerous white foci. Testicles-Normal Lung-green-black foci Kidney-normal large abscess at point of injection	Liver Spleen Lung Abscess	growth	growth	- - - - - - -	+	+	+	+	+	+
G.pig No.2 Vaccine 400R Bacterin 400S	Li. normal Sp.- white foci Testicles - normal Lung - greenish-black foci Kidney - normal Abscess at point of injection	Spleen Lung Abscess	Growth	No growth	+ + + + - - -	+	+	+	+	+	+
G.pig No.3 Vaccine 4R Bacterin 4S	Li. many pearly-white foci Sp. - enlarged Kidney - normal Lungs- normal Congested area on left side of the humerous, filled with fibrinous exudate	Liver Spleen Left anillary region	No growth	growth	+ + - - - - -	+	+	+	+	+	+

The data presented in table XVIII demonstrates the comparison of agglutination of R and RS antisera, when homologous rough and smooth types of antigens are used. It will be observed that the antisera obtained, as a result of injecting living R and killed S organisms into animals, produced antibodies for both the R and S antigen. The R antigen was agglutinated in low dilutions. The R antiserum contained antibodies for the R type antigen. It will be noted that the R type of Br. abortus was agglutinated by its own specific antiserum. R Br. suis and Br. melitensis are not agglutinated by R Br. abortus antisera.

TABLE XVIII. AGGLUTINATION TEST WITH GUINEA PIG ANTISERA.

Antigen	Antisera	C	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	1-5120	1-10240
400R	400R	-	++++	++++	++++	++++	++++	+++	+++	++	++	+
316R		-	++++	++++	++++	++++	+++	+++	++	++	+	+
4R		-	-	-	-	-	-	-	-	-	-	-
	316R											
400R		-	++++	++++	++++	++++	++++	++	++	+	-	-
316R		-	++++	++++	++++	++++	+++	+++	+++	++	++	+
4R		-	-	-	-	-	-	-	-	-	-	-
	4R											
400R		-	-	-	-	-	-	-	-	-	-	-
316R		-	-	-	-	-	-	-	-	-	-	-
4R		-	++++	++++	++++	++++	+++	+++	++	++	++	+
	400R											
400S		-	++++	++++	++++	++++	+++	+++	+++	+++	+++	+
400R		-	+++	++	++	++	-	-	-	-	-	-
	316RS											
316S		-	+++	+++	+++	+++	+++	++	++	++	+	-
316R		-	++	++	-	-	-	-	-	-	-	-
	4RS											
4S		-	++++	++++	++++	++++	++++	+++	++	++	++	+
4R		-	++	++	++	-	-	-	-	-	-	-

S = smooth type antigen
R = rough type antigen
400 = Br. suis
316 = Br. melitensis
4 = Br. abortus

Discussion.

This study was undertaken as stated in the introduction, primarily to obtain R forms of the various species of the Brucella group. Information in regard to strains studied in this work was first obtained. The R forms obtained are markedly different than the S prototype and because of this, a thorough study of the strains to be used was made. The cultures in all instances were checked for purity. The various incitants used were evaluated and it was found that aging gave the best results. In all instances where dissociation to R forms was obtained the original stock culture was checked for purity and the experiment repeated. Similar R types were obtained in all cases. Although the R types were quite different in many respects from the S prototypes, still the fact that the R forms were always obtained, argues quite conclusively for their identity as R forms of Brucella. To further prove their relationship, the induced R types were reverted to the S forms by rapid transferring and animal passage. The induced S forms were identical in reaction to the stock strains. The reversion of the R forms proves conclusively that they were types of Brucella and not contaminations.

The cultural, morphological and physiological properties of the R strains were markedly from the original stock strain. These changes are quite interesting inasmuch as such variations, at least to such a marked degree, are unusual of R types. In the case of

the paratyphoid group, the differences are serological and cultural while the physiological reactions of the R and S types are identical.

The R forms of Brucella on the basis of the data presented show a grouping into two classes. Using hydrogen sulphide and cross-agglutination as a basis for classification, the strains of Br. abortus were separate from Br. suis and Br. melitensis.

In view of the fact that only two R strains of Br. abortus were obtained, the data presented is not sufficient to warrant drawing conclusions. More R strains of Br. abortus must be obtained and tested before one can satisfactorily classify the R members of this group into two classes.

When living R and killed S organisms are inoculated into animals the resulting antiserum will contain antibodies for both types of antigen. Typical lesions were obtained in each of the animals that received the living R and killed S homologous organisms. The animals receiving living R organisms did not show lesions. Due to the lack of time, this experimental work was not repeated. However, this is in agreement with Dawson's work on pneumococci.

Summary.

A study of stock strains of Br. abortus, Br. suis and Br. melitensis was made to determine their purity and cultural, physiological and morphological characters as related to colonial appearance or S properties. The strains selected for study were all S type organisms.

The S type organisms selected were treated with various dissociation incitents to induce the formation of rough types. The incitants used were one per cent lithium chloride, 10 per cent positive immune serum broth, 0.1 per cent phenolized broth and aging of cultures on liver infusion agar slants. The process of aging on the liver infusion agar slants was found to be the best method of inducing R types. The 0.1 per cent phenolized broth caused only one organism to dissociate. This particular organism was very susceptible to dissociating agents. The 10 per cent positive immune serum yielded several R types but much less so than the process of aging.

The R types obtained were isolated and studied using the same methods as was used with the stock cultures originally.

The R cultures were found to be markedly different morphologically, physiologically, serologically and culturally from the original S types. In general the R types agreed among themselves except for variation with Br. abortus on H_2S and agglutinin production.

The repeated isolation of these R forms from the same cultures warrants the assumption that these organisms are dissociates, although they bear little if any resemblance to the parent form.

The R type organisms obtained by dissociation were reverted to their original type by means of growing on 10 per cent R antiserum broth and by injecting the R organism alone and together with killed S organisms of the homologous type. Although the 10 per cent R antiserum broth caused a reversion, the animal passage, especially when killed S organisms were injected gave the best results. The reversion of all the R forms to their original type demonstrates that they were not contaminants but R dissociates of Brucella.

Conclusions.

1. Smooth strains of the Brucella group were dissociated to the R type.

2. Rough strains were reverted to the homologous smooth type by serial transferring on S antisera and by inoculating a suspension of living and killed organisms in the guinea pigs.

3. R strains differed morphologically, culturally and physiologically from their S prototypes.

The writer takes this opportunity of expressing his appreciation to those who aided him in this work; Doctor W. L. Mallmann under whose supervision this work was planned and carried out; Doctor I. F. Huddleson who furnished me with cultures and Mr. J. P. Torrey who was of great assistance in the examination of the autopsied experimental animals.

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Gallo