THE EFFECT OF SERUM UPON DISSOCIATION IN BRUCELLA ABORTUS: A DEMONSTRATION OF THE ROLE OF SELECTIVE ENVIRONMENTS IN BACTERIAL VARIATION[1]

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A previous report (Braun, 1946) on studies with *Brucella abortus*, strain 19, led to an interpretation of dissociation in terms of spontaneous appearance of variants and their subsequent establishment under the control of inherent and environmental factors governing population dynamics. One outgrowth of these studies was an attempt to produce *Brucella abortus* vaccine from smooth clones with low dissociation index (D.I. = the percentage of dissociated types within a population after 10 days of growth in broth; for details see Braun, 1946). In the course of these additional studies it was observed that vaccines made from some clones were superior in certain characteristics to control vaccines made from Bureau of Animal Industry strains; but it was also found that the intense growth period on Blake bottles, necessary in the manufacture of vaccines, can produce a change in the selected inherent characteristics of the material used by permitting the establishment of smooth organisms with changed dissociation indices. Therefore, it became desirable to search for means to prevent changes in growing populations, i.e., an environment had to be found which would permit the propagation of the selected type only and would prevent the establishment of changed types (mutants). It was thought that such selective environments might be produced by adding to cultures material containing antibodies for types which are not desired.

Previous *in vitro* studies by Dawson and Sia (1931) and Alloway (1932) have demonstrated that R types of pneumococcus can be "transformed" to S types if grown in the presence of anti-R serum, or in the presence of normal swine serum, which according to Alloway contains R antibodies. (Avery, MacLeod, and McCarty, 1944, later obtained results which suggested that factors other than R antibodies are involved.) The following experiments were, therefore, designed to investigate the effect of certain antisera and normal sera upon the dissociation of *Brucella abortus*.

EXPERIMENTAL[2]

For the production of antisera, rabbits were inoculated with suspensions of either smooth, rough, brown, or a mixture of rough and brown, all of which had originated from the progeny of a transplant of strain 19-12A of *Brucella abortus*,

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[2] For general procedures see Braun, 1946.

which had been received from the Bureau of Animal Industry on October 19, 1943. Inoculations were made weekly over a period of 3 weeks. One week after

TABLE 1

Summarized tabulation of the effects of serum or plasma upon dissociation

STRAIN USED	TYPE OF SERUM OR PLASMA ADDED TO BROTH CULTURES	AMOUNT ADDED TO 5 ML OF BROTH	AVERAGE D.I. PER CENT	NUMBER OF CULTURES
S, Clone 2583	none	none	20	48
	Normal serum from *rabbit*	0.05 to 1.0 ml	0†	60‡
	R antiserum from *rabbit*			
	Br antiserum from *rabbit*			
	R + Br antiserum from *rabbit*			
	S antiserum from *rabbit*	0.05 to 1.0 ml	2	8
	Normal serum from *cow*	0.1 to 1.0 ml	0	58
	Normal plasma from *cow*	0.1 to 1.0 ml	0	16
	Normal serum from *hog*	0.1 to 1.0 ml	0	6
	Normal serum from *goat*	0.5 ml	0	4
	none*	none	61	2
	Normal serum from *cow**	0.2 to 0.5 ml	0	7§
S, Clone 2907	none	none	20	6
	R antiserum from *rabbit*	0.05 to 1.0 ml	0¶	21‖
	Br antiserum from *rabbit*			
	R + Br antiserum from *rabbit*			
	S antiserum from *rabbit*	0.05 to 1.0 ml	4	8
S, 19–17 A (B.A.I. culture)	none	none	29	6
	R antiserum from *rabbit*	0.05 to 1.0 ml	0	20
	Br antiserum from *rabbit*			
	R + Br antiserum from *rabbit*			
	S antiserum from *rabbit*	0.05 to 1.0 ml	<1	8

Total number of cultures with serum (or plasma): 221.

Number of cultures showing dissociation in the presence of more than 2 per cent serum or plasma (except S serum): 4.

* Kept at 38 C.

† D.I. of 2 cultures with 0.05 ml Br antiserum added: less than 1 per cent.

‡ D.I. of 1 *additional* culture with 0.5 ml Br antiserum: 12 per cent. D.I. of 1 *additional* culture with 0.5 ml R + Br antiserum: 4 per cent. D.I. of 1 *additional* culture with 0.1 ml R + Br antiserum: 3 per cent.

§ D.I. of 1 *additional* culture with 0.5 ml serum added: 7 per cent.

¶ D.I. of 2 cultures with 0.5 ml R + Br antiserum added: less than 1 per cent.

‖ D.I. of 1 *additional* culture with 0.05 ml R antiserum: 12 per cent.

the last inoculation the rabbits were bled from the heart.[3] The antisera were then added in varying amounts to broth cultures, which were inoculated with

[3] In agglutination tests rough and brown antigens exhibited a definite reaction with all antisera used. This reaction differed somewhat from the typical agglutination observed when S antigen and positive S antiserum were used, i.e., the agglutination was less pronounced, but nevertheless significantly different from the settling of control antigens without antiserum.

equal amounts of a suspension of clone 2583, a clone with a fairly high dissociation index under standard conditions. Table 1 summarizes the results of a series of such experiments. Except for a very small number of cultures (1 in 60), all cultures to which antiserum of rough or brown, or even normal serum, had been added showed absence of dissociation, but control cultures without serum showed considerable dissociation. The addition of serum, in concentrations as low as 2 per cent, thus usually suppresses the establishment of dissociated types. It appeared unnecessary to produce specific antisera against rough or brown,because the initial results indicated that normal serum contains factors which suppress rough and brown types. This was further confirmed in extensive tests (summarized in table 1), which revealed that normal serum or plasma from cows, as well as from hogs and goats, suppress the establishment of rough and brown types efficiently.[4] (The presence in normal serum of factors reacting with rough and brown types is also supported by the reaction observed in agglutination tests.)[5] The addition of 0.01 ml or less of serum or plasma to 5 ml of broth did not prevent dissociation.

In the case of the addition of S antiserum (table 1) some dissociated types do establish themselves; presumably the suppression of S types due to the presence of S agglutinins is stronger than the suppression of rough and brown types by factors normally present in serum.

In the rare instances in which dissociated types occur in the presence of more than 2 per cent serum, the variants usually differ from the types commonly observed. A "whitish brown" type (mucoid), a "transparent" type (mucoid), and a "brownish rough" type have been isolated from such exceptional cultures. Presumably these uncommon variants cannot establish themselves when they are in competition with arising rough and brown types. Only when the establishment of the latter types is prevented through the factors present in serum do the uncommon types attain a positive selection value which permits their establishment.

Recently, Huddleson *et al.* (1945) published a detailed report dealing with the bactericidal action of bovine serum and plasma on *Brucella abortus*. Although the concentration of serum in the experiments reported in the present paper was very much smaller than the concentrations used by Huddleson *et al.* for the demonstration of bactericidal activity, it appeared necessary to test whether our results might be due to the bactericidal action of serum or plasma. Such tests appeared to be of special significance since it had been previously shown that factors which affect growth-rates also affect dissociation (Braun, 1946). The absence of dissociation in cultures to which serum has been added could, therefore, be due merely to a general retardation of growth. Experiments designed to test the possible relationship between the general bactericidal activity of serum

[4] Horse serum and chicken serum do not suppress dissociation so effectively as sera from other animals tested. Uncommon types of variants were found in many cultures to which chicken serum had been added.

[5] See footnote 3.

and the suppression of dissociation, however, revealed that the suppression of dissociation in cultures with serum is not due to any general bactericidal or bacteriostatic acitivity. First, it was found that the growth rates of smooth types were not affected by the addition of serum sufficient for the suppression of dissociated types (table 2). Second, Huddleson *et al.* had demonstrated that the bactericidal activity depends on the presence of complement and that the removal of complement by heating at 56 C for 1 hour, or by filtration through a Berkefeld W filter, removed the bactericidal action. Results compiled in table 3 show that

TABLE 2

Growth in the presence and absence of serum

	AT START OF CULTURE	AFTER 3 DAYS	AFTER 10 DAYS	
Total number of cells*	231 M†	460 M	1.450 B‡	Without serum
Total number of cells*	231 M	488 M	1.413 B	With 0.2 ml normal rabbit serum
Total number of cells*	231 M	478 M	1.500 B	With 0.2 ml normal cow serum

* All figures are averages of 4 cultures.
† Millions.
‡ Billions.

TABLE 3

The effects of filtered or heated serum upon dissociation

STRAIN USED	TYPE OF SERUM ADDED TO BROTH CULTURES	AMOUNT ADDED TO 5 ML OF BROTH	AVERAGE D.I. PER CENT	NUMBER OF CULTURES
S, clone 2583	none	none	20	48
S, clone 2583	Normal serum from cow, *filtered* through Berkefeld W filter	0.1 ml, *or* 0.2 ml, *or* 0.5 ml	0	12
S, clone 2583, 2907, 1863, & strain 19–17A	Normal and antisera from rabbit, *heated* 1 hour at 56 C	0,1 ml, *or* 0.2 ml, *or* 0.5 ml	0	50
S, clone 2583	Normal serum from cow, *heated* for 30 min at 62 to 63 C	0.5 ml	0	8

neither heating nor filtration removed the factors responsible for the suppression of dissociated types in our experiments.

It could be assumed that the factors present in serum and plasma either prevent the change from smooth to rough or brown, or inhibit the establishment of the latter types, or that they do both. Whereas it has not been possible yet to investigate the first-mentioned possibility, it was proved by a series of tests that the establishment of rough and brown types is actually inhibited in the presence of serum. Cultures with and without serum were started with known percentages of smooth and rough organisms. Ten days later samples from these cultures were streaked on "2–1 agar" plates, and the percentages of smooth and rough

colonies was determined. No increase in the percentage of roughs was found in cultures containing serum, whereas control cultures without serum showed a considerable increase of the percentage of roughs over smooth (table 4).

So far all attempts to suppress the growth of dissociated types on solid media, through the addition of serum to "2–1 agar," have failed. It has also been observed that the addition of 10 per cent of normal serum from nonvaccinated, noninfected cows to vaccines not only prevented the occurrence of dissociation but also caused a significant increase in viability during storage.

TABLE 4

The inhibitory effect of normal cow serum upon the establishment of R types

AMOUNT OF NORMAL COW SERUM ADDED PER BROTH CULTURES	PERCENTAGE OF ROUGH AMONG SMOOTH	
	At start of broth cultures	After 10 days
None	1	22
0.2 ml	1	<1
None	5	24
0.2 ml	5	3

DISCUSSION

The observed suppression of rough and brown variants in the presence of serum or plasma demonstrates the selective role of environmental conditions in bacterial variation. Under standard *in vitro* conditions, i.e., buffered broth,[6] rough and brown variants can establish themselves within an originally smooth population because of their greater viability (Braun, 1946). Under standard *in vitro* conditions rough and brown variants, therefore, have a higher selection value than smooth. The addition of serum or plasma obviously alters this selection value. Conditions similar to those observed when serum is added to broth appear to exist *in vivo*, where it has been observed that the S type only can be isolated after inoculation of a mixture of R and S variants (Henry, 1933). The environment thus has a profound effect on the "pattern" of bacterial variation by determining the selection value of variants that arise, regardless of the inherent tendencies for dissociation.

In this connection the problem of apparently successive orderly changes ("cycles") in bacterial variation may be discussed, a problem which has contributed much to what can now be considered previous misinterpretations of basic aspects of dissociation. The information now available makes it clear that of all spontaneously arising variants only those can establish themselves which have a higher selection value (growth rate or viability) than the original members of the population. For example, of all mutants which can occur within a smooth population rough and brown types usually have the highest selection value under standard *in vitro* conditions. According to some unpublished experimental observations on variants which arose from one clone, smooth types have the fastest growth rate, rough types a slower growth rate, and brown types a still slower growth rate; brown types have the highest viability (i.e., ratio of total number of

[6] Beef extract broth.

cells to viable number of cells per ml of broth), rough types a slightly lower viability, and smooth types have the lowest viability. Since the number of viable cells per ml is limited (Braun, 1946), rough and brown mutants which arise in the smooth population will replace the smooth type; during prolonged growth rough types will become predominant first and they will eventually be replaced by the brown type, causing an apparently orderly change from S → R → Br. Thus, competition between spontaneously arising mutants with different selection values, leading to the establishment of one predominant type (i.e., the one which shows highest viability under any given environmental conditions), will produce the appearance of successive orderly changes. Furthermore, since the rough type has only been able to establish itself within an originally smooth population through its higher viability, it now becomes obvious why it is usually so difficult to observe dissociation from rough to smooth *in vitro*. Should one smooth mutant arise in a rough population (as it probably does), it will have little chance to establish itself because it is competing with members of a population which have already a higher viability than the smooth mutant. If, however, environmental conditions are changed so that the smooth type attains a higher selection value than the rough type, as is the case in the presence of serum, then a change from rough to smooth can occur. In addition, certain smooth mutants can establish themselves in a rough population under standard *in vitro* conditions, namely, smooth mutants which have a higher selection value than members of the particular rough population in which they arise. Such smooth mutants have been observed (Braun, 1946).

The ability of spontaneously arising variants with positive selection value to establish themselves rapidly, within a population with lesser selection value under given environmental conditions, can be held responsible for the apparently specific "adaptation" which can be observed among bacteria under natural conditions, and can account for the occurrence of considerable variation when bacteria are removed from their normal environment and are grown under laboratory conditions. Removal from their normal environment apparently alters the selection value of arising variants; members of the original population cease to be the ones with greatest viability or growth rate, and arising mutants which formerly were unable to compete with the "normal population" can now establish themselves. The so-called "normal type" is thus merely one variant which has been able to establish itself under natural conditions in response to the forces of selection.

SUMMARY

Serum or plasma of normal cows, rabbits, hogs, and goats was found to contain factors which suppress the establishment of rough and brown variants of *Brucella abortus*. When small amounts of serum or plasma were added to smooth broth cultures, which normally would show considerable dissociation after 10 days, no dissociation occurred.

The suppression of dissociated types by serum or plasma is not due to factors which are responsible for the bactericidal action of sera or plasma.

These results form the basis for a discussion of the selective role of environments in bacterial variation, the appearance of *apparently* successive orderly changes during dissociation, the difficulties encountered in reversing the direction of dissociative changes, and the apparently specific adaptation of bacteria under natural conditions.

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