

THE EFFECT OF DISSOCIATION ON THE ELECTROPHORETIC MOBILITY OF BRUCELLA^{1,2,3}

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Received for publication March 31, 1941

Electrophoretic investigations of rough and smooth forms of bacteria have been confined largely to a few groups of organisms. Brown and Broom (1929) and Ahuja (1929) reported there was no difference in electrophoretic mobility of R and S forms of intestinal bacteria; however, Joffe, Hitchcock, and Mudd (1933), Joffe and Mudd (1935), and Moyer (1936b) have shown that R and S forms of these organisms have mobilities that are quite different from each other. Appreciable differences in mobility of R and S forms of tubercle bacilli were observed by Kahn and Schwarzkopf (1930), Reed and Gardiner (1932), (1934), and Choucroun and Plotz (1934). Electrophoresis studies on vibrios, both R and S forms, have been especially investigated by Soru (1934) and Linton, Mitra, and Seal (1938). These last authors conclude that electrophoretic data give a quantitative measure of the roughness of organisms. Rane (1929) observed differences in electrophoretic behavior of R and S forms of *Bacterium phaseoli* (*sojense*). Thompson (1931) reported differences in electro-

¹ Paper No. 1896 Scientific Journal Series, Minnesota Agricultural Experiment Station.

² The investigation herein reported was in part made possible through support granted by the Bureau of Animal Industry, United States Department of Agriculture.

³ The material for this paper was taken from a thesis submitted by Dr. Stearns to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy, March, 1940.

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phoretic mobility of R and S forms for some pneumococci but not for others. The report of Moyer appears to be the only one showing quantitative data on the electrophoretic behavior of mixed cultures of R and S forms under standard conditions.

Having investigated various factors involved in the electrophoretic behavior of *Brucella* and having observed large differences in electrophoretic mobility between R and S forms of these organisms, (Stearns and Roepke, 1941), it was thought to be of interest to follow surface changes occurring during dissociation by the determination of electrophoretic mobility.

METHODS

Electrophoretic measurements were made in the same Abramson microelectrophoresis cell used in the first of this series of papers, following the technique described in detail by Moyer (1936a). Current intensity through the cell was kept constant throughout the measurements on suspensions, and the electrophoretic mobilities were calculated by the use of Ohm's law. Measurements were made as objectively as possible by timing the first organism seen in the microscopic field. Readings were made with at least two settings at each of the stationary levels located at 20.1 per cent and 79.9 per cent of the depth of the cell for the particular electrophoresis cell used. More details of the electrophoretic technique used, as well as a criticism of the literature on electrophoresis of bacteria, are given by Stearns and Roepke (1941).

Investigations were carried out on four smooth laboratory strains of *Brucella abortus*, of which strains 76A, 62, and 281 have been in use in this laboratory for several years for antigen production, while strain 19, of low virulence but of high antigenic character, is used in vaccination studies.

Inoculations were made into nutrient broth at pH 6.8, then after various time intervals of incubation they were transferred into fresh broth through several successive transfers. All suspensions for electrophoretic measurement were prepared by inoculating organisms from a given transfer in broth onto potato agar slants. After 3 days' incubation the organisms were har-

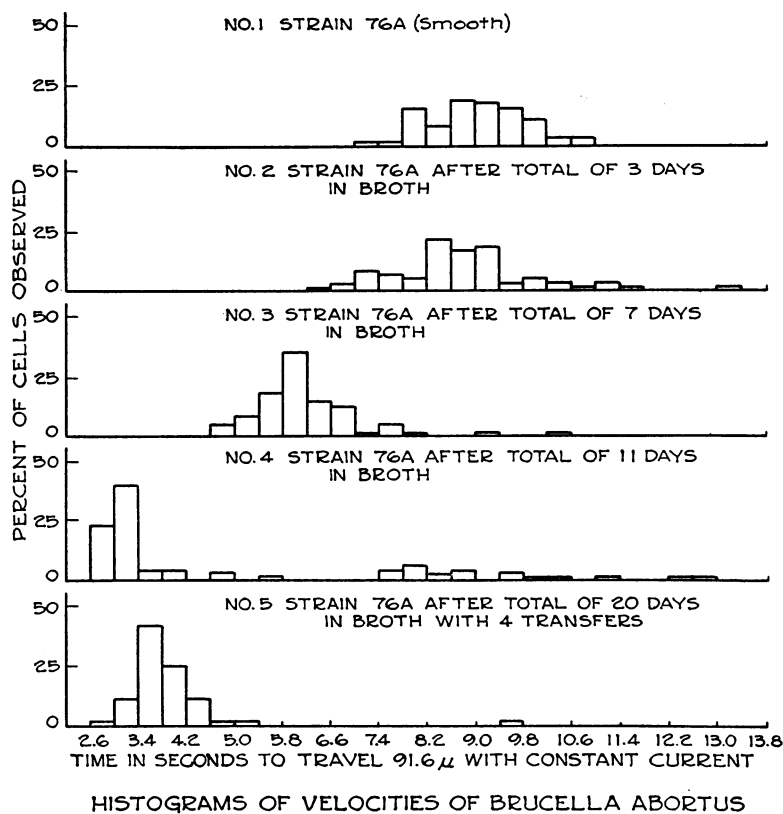
vested. The organisms were washed three times with physiological saline, after which they were killed by contact with 0.2 per cent formaldehyde. The use of formaldehyde was found to be without effect on the mobility of the organisms, (Stearns and Roepke, 1941). For mobility measurements, 0.01 ml. of a dense washed suspension of formaldehyde-killed organisms was added to 25 ml. of 0.01 M. acetate buffer at pH 4.7. It is to be understood that all organisms studied throughout this investigation were negatively charged under the conditions employed. Further details are described in the experiment.

EXPERIMENTAL RESULTS

Smooth strains 76A and 281 were inoculated into nutrient broth in duplicate and electrophoretic measurements made on suspensions prepared as described above. Measurements were obtained on the effect of 3, 7 and 11 days in broth, and 20 days in broth with four transfers. Since data obtained on strain 281 were similar to those of strain 76A, with the exception that greater difficulty was experienced in producing dissociation, only the results on the latter strain are presented in figure 1. Results are shown in the form of frequency histograms in order to present advantageously the range of mobilities of organisms. From 56 to 78 organisms were timed for each suspension. The histograms have, as a base line, time in seconds for the organisms to traverse 91.6 microns, when the current intensity is 0.6 m.a. which gives a field of 7.0 volts per centimeter at 25°C. Time values are plotted rather than mobilities, since mobility varies inversely with time values. This avoids weighting the data at the extremes and avoids the resulting appearance of skew, (Moyer, 1936b).

At first, growth in the broth was diffuse, with the organism showing little tendency to settle out. After 3 or 4 days' growth, dissociated forms began to appear, exhibiting mucoid character and collecting in stringy masses on the bottom and surface of the broth. These variants have been termed rough forms by some authors, but they do not conform to characteristics of the R form of *Brucella* described by Mallmann and Gallo (1933). Plastringe and McAlpine (1930) describe this mucoid form, and Mickle

(1940) reports that this form is encapsulated. After several days in broth, most of the growth was confined to a large sticky clump at the bottom of each tube. Great difficulty was experienced in suspending the more dissociated forms. During the



HISTOGRAMS OF VELOCITIES OF BRUCELLA ABORTUS

Fig. 1

washing procedure after each centrifugalization and decantation, these organisms were rubbed vigorously with a glass rod to aid in

In figure 1, histogram 1 gives some idea of the normal spread of mobilities of cells of a smooth strain grown on a medium not causing dissociation. Although there was a slight increase in the range of mobilities after 3 days in broth, shown in histogram 2, the first clear electrophoretic indications of dissociation occurred

after 7 days in broth, histogram 3. This histogram shows that a definite shift of the mean mobility has occurred. A few of the organisms have mobilities comparable to the original smooth organisms; however, the mode is shifted toward the dissociated forms, i.e., higher mobility.

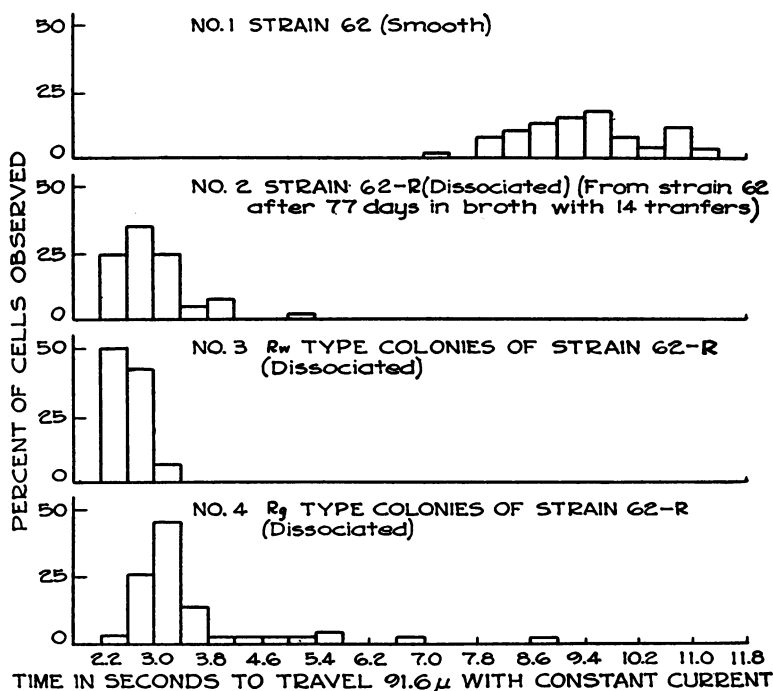
In histogram 4 is shown the spread of mobilities after 11 days in broth. With the further shift of some organisms toward higher mobilities (lower time values), a separation of the organisms into two mobility modes may be observed, one having mobilities the same as the original mode. Smith and Joffe (1934), in their study on *Brucella*, report considerable variation of electrophoretic mobility of individual bacteria in a given culture and that some subcultures occasionally have mobilities different from the original. If work were begun on a strain in the condition depicted in histogram 4, that is, partly dissociated, it might show results similar to those obtained by Smith and Joffe. No data were obtained here as to the stability of this partially dissociated form with continued transfers on solid media. The data here emphasize the necessity of establishing the homogeneity of a culture before beginning electrophoretic investigations.

Separation of dissociated forms

Smooth strains 62 and 19 were inoculated into broth in duplicate and kept in broth for 77 days with fourteen transfers. Washed suspensions were prepared in the usual manner from 3-day cultures on potato agar inoculated from the last transfer in broth, and mobilities were determined. The fourteenth transfer in broth of strain 62 (designated 62-R) was plated on potato agar and after 4 days examined under a dissecting microscope with oblique transmitted light in the manner described by Henry (1933) for the selection of smooth and rough colonies of *Brucella*. By this method, culture 62-R showed the presence of two types of colonies: one type appeared white in color and the other type grey in color.

Three of the white colonies (designated 62-Rw) and two of the grey colonies (designated 62-Rg) were selected for cultures on potato agar and mobilities studies. The mobility data obtained

on cultures of the Rw and Rg types of colonies are shown in figure 2 in the form of histograms. Since the mobilities of the organisms from suspensions prepared from the Rw colonies had the same mobility range, the data are grouped together in histogram 3. Similarly, the data on the organisms of suspensions prepared from the two Rg colonies are grouped together in histo-



HISTOGRAMS OF VELOCITIES OF BRUCELLA ABORTUS

FIG. 2

gram 4. For comparison, the spread of mobilities of the normal smooth strain 62 is also included. Strain 62-R refers to strain 62 after the passage through broth, from which the Rw and Rg types of colonies were selected. From 56 to 120 organisms were timed for each measurement on a suspension.

Histograms 1 and 2 show the same large shift in the mobility range of organisms upon passage through broth as was shown by

strain 76A in figure 1; similar changes occurred with strain 19. The data shown in histograms 3 and 4 seem to indicate that the technique frequently used for selecting rough and smooth colonies of *Brucella abortus* may at times yield a separation of organisms dissociated to different degrees. This may appear more clear if the mobilities are compared in microns per sec. per volt per cm. The mobility, calculated from the mean time value, of the organisms from the Rw type (histogram 3) is 2.82, that from the Rg type (histogram 4) is 2.24 and the mobility of the suspension from which the two types were derived (histogram 2) is 2.35 microns per sec. per volt per cm., all values calculated to 25°C. Although a few of the organisms shown in histogram 4 had mobilities comparable to those of the original smooth strain 62, it seems likely that the appearance of the colony would be determined largely by the organisms of higher mobility which account for almost all of the organisms in the colony. Cultures of both the Rw and Rg colonies were mucoid in character, as evidenced by the slimy or stringy nature of suspensions prepared from the cultures.

Passage of dissociated strain 62-R through guinea pigs

A suspension of the mucoid strain 62-R of high mobility used in the preceding experiment was inoculated into three guinea pigs, which after 5 weeks were killed and their spleens cultured. *Brucella* organisms were isolated from all of the three guinea pigs (these cultures were designated 62-R'-1, 62-R'-2, 62-R'-3). Serum from one of the guinea pigs agglutinated normal polyvalent smooth antigen in 1:25 dilution. Sera from the other two did not agglutinate the antigen in this or higher dilutions. When suspension of the organisms isolated from the guinea pigs were prepared in the manner described, they had mobilities in the acetate buffer comparable to that of the original smooth strain 62 when recently passed through guinea pigs; however, the organisms were still in the mucoid phase. It would thus appear that the outermost surface of the mucoid organisms had undergone a physical or chemical change during passage through guinea pigs.

For serological studies on the above low mobility mucoid type organisms, antisera were prepared by inoculating guinea pigs with the live organisms of cultures 62-R'-1, 62-R'-2, 62-R'-3 and the original smooth strain 62. The guinea pigs were killed 6 weeks later, their sera collected and the spleens cultured. Organisms were isolated from only one of the guinea pigs inoculated with the low mobility mucoid type cultures and from the guinea pig inoculated with the smooth strain 62. A photoelectric turbidometer was used to adjust the density of the various antigens used in the agglutination tests to the density of the normal polyvalent antigen routinely used in testing for Bang's disease.

TABLE 1
Serological studies on smooth strains and several rough strains of Brucella abortus

ANTISERA	ANTIGENS					
	62-R'-1	62-R'-2	62-R'-3	62-R	62 (Smooth)	Smooth Polyvalent
62-R'-1	+-----	+++++I-	++I----	+++I----	-----	-----
62-R'-2	II-----	+++++--	+++I----	+++I----	-----	-----
62-R'-3	++I----	+++++++	++++++I	+++I----	-----	-----
62 (Smooth)	+++++++	+++++++	+++++++	+++++++	+++++++	++++++I
Negative		II-----	??-----	++I----	-----	

Dilutions of sera = 1:25, 1:50, 1:100, 1:250, 1:500 and 1:1000. + = complete agglutination. I = incomplete agglutination. - = no agglutination.

Negative guinea pig serum was used as a control. The results of the agglutination tests are shown in table 1.

The antigens prepared from the rough strains 62-R, 62-R'-1, 62-R'-2 and 62-R'-3 for the agglutination tests were not very satisfactory because of the instability of the suspensions. It was necessary to suspend the mucoid type of growth by shaking with glass beads in a shaking machine. The instability of the suspensions is shown by the results obtained with negative serum.

Although the strains 62-R'-1, 62-R'-2 and 62-R'-3 obtained by the passage of the rough strain 62-R through guinea pigs showed a mobility the same as the smooth strain 62, the cultures were of the mucoid type and gave agglutination reactions very similar to the high mobility mucoid strain 62-R. Thus, the marked differ-

ences in mobility between the first three antigen strains of table 1 and strain 62-R would indicate very definite differences in the nature of the cell surfaces of the two mucoid forms, whereas the agglutination reactions indicate a similarity in the surfaces of the two mucoid forms. Apparently the factor or factors which affect the mobility or electrokinetic properties of the cell surface are not necessarily the same as those which are responsible for the antigenic properties.

The rough strains 62-R'-1, 62-R'-2 and 62-R'-3 were agglutinated by high dilutions of antiserum prepared from the smooth strain 62, but did not cause the production of significant amounts of antibodies for the smooth strains.

DISCUSSION

This study emphasizes the great complexity of the field of dissociation. Although electrophoretic measurements may not reflect all changes that occur during dissociation, following the course of dissociation by observations of the changes in the electrokinetic potential with respect to a given medium may offer a more refined technique for observing dissociation, especially in its earlier stages.

The data suggest that dissociation is a gradual process; not only may dissociants gradually appear while there are smooth organisms still present, but also the first dissociants may continue to change toward more completely dissociated forms. Further study is indicated along the line of separating various intermediate forms with tests of their stability. In this connection, Hadley (1937) has stated that it is not evident that all species should fit in with the three-phase system of designating dissociants (rough, smooth, and mucoid).

Too few data have been obtained in this study to make any broad generalizations; however, the results obtained here and the results given in the previous paper on variation of mobility of rough, smooth, and mucoid forms with pH, suggest that electrophoretic measurements may give an added criterion for separating these forms.

SUMMARY

1. Electrophoretic measurements under carefully controlled conditions on strains of *Brucella abortus* dissociated in broth indicate that dissociation may be a gradual process. Dissociated forms (mucoid) having a greater mobility than the smooth appear after a few days in broth without the complete disappearance of organisms having the mobility values of smooth forms. Longer passage in broth causes further increases in mobility of dissociated forms and the gradual disappearance of organisms of mobility comparable to that of smooth strains.

2. Dissociated forms having appreciably different mobility values may be separated by colony appearance when a given technique is used. The stability of these dissociated forms was not investigated.

3. Passage of dissociated forms of *Brucella* of high mobility through guinea pigs causes a decrease in mobility value to that of the smooth forms without the loss of mucoid character. Serological tests show that mucoid organisms of low mobility are similar antigenically to mucoid forms of high mobility, but are appreciably different from the original smooth organisms.

4. The complexity of the subject of dissociation is emphasized and lines of further investigation are indicated.

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